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PROCOAGULANT CHANGES IN AIR POLLUTION

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PhD dissertation submitted in fulfillment of the requirements to obtain the degree of Doctor in Biomedical Sciences.

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Katholieke Universiteit Leuven
Group Biomedical Sciences
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Il y a des moments où tout réussit.
Il ne faut pas s'effrayer, ça passe.

(Jules Renard)

Cover: "Carbon pollution" by Katrien Falaise

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TABLE OF CONTENTS:

LIST OF ABBREVIATIONS

95%CI	95% confidence interval
ACE-inhibitor	Angiotensin-converting enzyme inhibitor
APHEA	Air Pollution and Health: A European Approach
APHENA	Air Pollution and Health: A Combined European and North American Approach
aPTT	Activated partial thromboplastin time
AT	Antithrombin
AV	Annexin V
AV^+ μV	Annexin V-binding microvesicle
BALF	Bronchoalveolar lavage fluid
BMI	Body mass index
BP	Blood platelet
BP μV	Blood platelet-derived microvesicle
CAP	Concentrated ambient particles
CIMT	Carotid intima-media thickness
CRP	C-reactive protein
DEP	Diesel exhaust particles
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immuno sorbent assay
ETP	Endogenous thrombin potential
FVII	Factor VII
FVIII	Factor VIII
FXII	Factor XII
Fbg	Fibrinogen
Hgb	Hemoglobin
IL-1β	Interleukin 1β
IL-6	Interleukin-6
IQR	Interquartile range
KC	Keratinocyte-derived chemokine
LDL	Low-density lipoprotein
LMWH	Low molecular weight heparin
LPS	Lipopolysaccharide
MCP-1	monocyte chemotactic protein-1
MIP-1a	Macrophage inflammatory protein-1α
MP	Microparticle (also called 'microvesicle')
μV	Microvesicle (also called 'microparticle')
NMMAPS	National Morbidity and Mortality Air Pollution Study
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_x	Nitrogen oxides
O_3	Ozone
ox-LDL	Oxidized low-density lipoprotein
P25	Percentile 25
P75	Percentile 75
PAI-1	Plasminogen activator inhibitor 1
PC	Protein C

PF4	Platelet factor 4
PFA-100	Platelet Function Analyzer-100
PM	Particulate matter
$PM_{0.1}$	Particulate matter with a mean aerodynamic diameter smaller than 0.1 μm
$PM_{2.5}$	Particulate matter with a mean aerodynamic diameter smaller than 2.5 μm
PM_{10}	Particulate matter with a mean aerodynamic diameter smaller than 10 μm
PMP	Blood platelet-derived microparticle
P-sel	P-selectin
PT	Prothrombin time
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RBC	Red blood cells
RBC-MP	Red blood cell-derived microparticle
RBC μV	Red blood cell-derived microvesicle
SD	Standard deviation
sE-sel	Soluble E-selectin
SO_2	Sulphur dioxide
sP-sel	Soluble P-selectin
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
TGA	Thrombin generation assay
TNFα	Tumor necrosis factor α
UPM	Urban particulate matter
UFP	Ultrafine particles
VTE	Venous thromboembolism
VWF	Von Willebrand factor
VWFag	Von Willebrand factor antigen
WBC	White blood cells

INTRODUCTION

Adapted from

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and

Emmerechts J & Hoylaerts MF. The effect of air pollution on hemostasis. Hamostaseologie 2011; 32, DOI: http://dx.doi.org/10.5482/ha-1179, epub ahead of print

Air pollution

Ambient environmental air pollutants include gaseous (carbon monoxide, nitrogen oxides, sulfur dioxide, ozone) and particulate components. The particulate component, particulate matter (PM), consists of a heterogeneous mixture of solid and liquid particles suspended in air, and is subdivided based on size ranges into 'thoracic particles' (PM_{10}, with a mean aerodynamic diameter <10 μm), 'coarse particles' (>2.5 μm and <10 μm), 'fine particles' ($PM_{2.5}$, <2.5 μm), and ultrafine particles (UFP, <0.1 μm) (Fig. 1). Although exposure to some gaseous components has been linked to cardiovascular events, the larger body of evidence points towards the deleterious effects of the particulates in polluted air.

Primary particles are emitted directly into the atmosphere, such as diesel soot, whereas secondary particles are created through physicochemical transformation of gases, such as nitrate and sulfate formation from gaseous nitric acid and sulfur dioxide (SO_2), respectively.

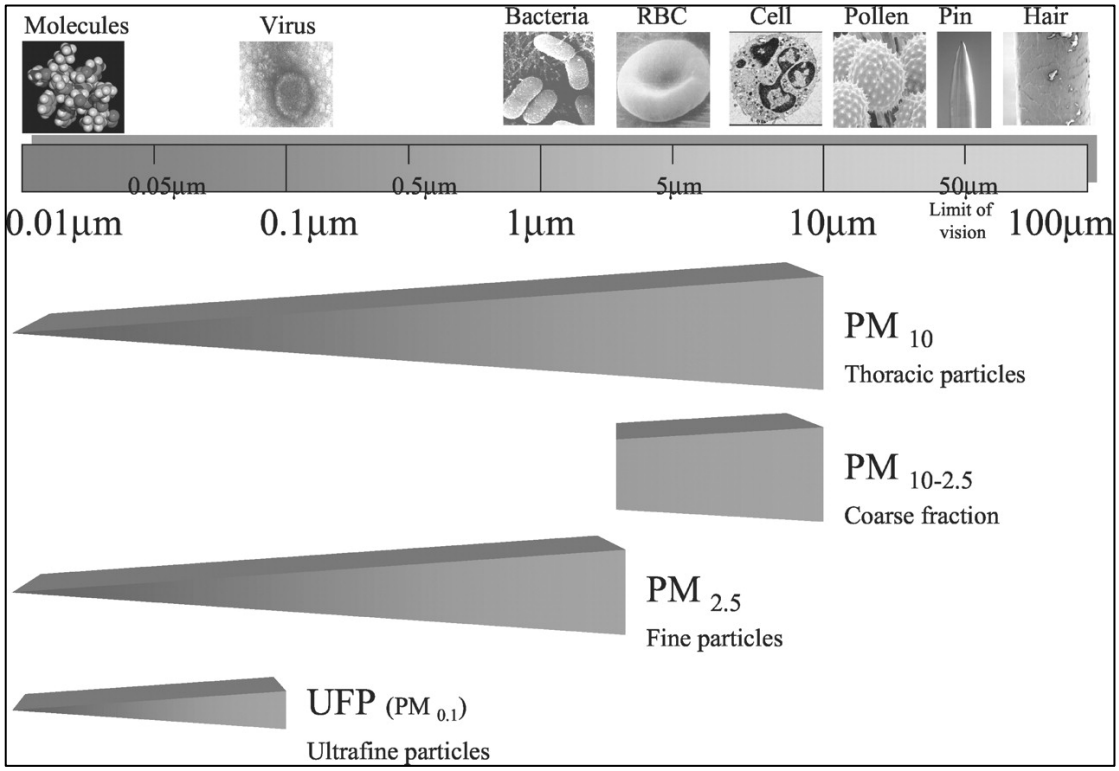

Figure 1. Size distribution of PM air pollution (from (1), with permission)

The numerous natural and anthropogenic sources of PM include motor vehicle emissions, tire fragmentation and resuspension of road dust, electric power generation and other industrial combustion, agriculture, construction and demolition activities,

residential wood burning, windblown soil, pollen and molds, forest fires, volcanic emissions and sea spray.
In general, $PM_{2.5}$ originates mostly from combustion sources, whereas the coarse fraction derives predominantly from natural sources, especially crustal material (including windblown soil) and grinding processes. Important bioaerosols (eg. endotoxin, pollen grains and fungal spores) are found mostly in the coarse fraction (and larger particles).

Generally, larger particles demonstrate a greater fractional deposition in the extrathoracic and upper tracheobronchial regions, whereas smaller particles (eg. $PM_{2.5}$) show greater deposition in the deep lung. More recently, considerable research attention has been devoted to the UFP, which, by virtue of their small size (<0.1 μm) penetrate deeply into the lungs and deposit in the alveoli. UFPs tend to be short-lived, because they agglomerate and coalesce into larger particles. They account for a major portion of the actual numbers of particles within PM, and have a high surface area-to-mass ratio, potentially leading to enhanced biological toxicity, in spite of their limited mass (1).

Air pollution and cardiovascular disease

Introduction

The human cardiovascular system consists of a functional vascular network for blood distribution, subdivided in a systemic and pulmonary circulatory system. The systemic circulation transports oxygenated blood through the arteries from the left heart to the organs and returns oxygen-depleted blood through the veins to the lungs. The pulmonary circulation subsequently transports the oxygen-depleted blood from the heart to the lungs, where it is oxygenated and returned to the heart.
Vascular integrity throughout the vascular tree is maintained by the vessel wall itself, as well as by a complex hemostatic mechanism with different protagonists for the arterial and the venous vasculature, involving blood platelets and coagulation factors.

The critical need to rapidly form a stable, localized clot in response to vascular injury (='hemostasis') must be balanced with the need to maintain blood flow within the

vessels. Different antihemostatic mechanisms prevent clot formation under resting physiological conditions, and limit clot growth to the site of vascular injury. When prohemostatic tendencies proceed beyond the physiological need to maintain vascular integrity, a pathological thrombus may form, obstructing the normal blood flow (='thrombosis'). In the arterial system, thrombus formation induces oxygen-deprivation (ischemia) of the downstream tissues, such as myocardial infarction and cerebral ischemia. The formation of an arterial thrombus largely depends on the activation of blood platelets, and is most often triggered by the rupture of an atherosclerotic plaque. Indeed, the chronic localized deposition of lipids into the arterial vessel wall (atherosclerosis) leads to the formation of plaques that can rupture when unstable, hereby exposing their procoagulant contents to the circulation (2). Hence, while often being asymptomatic in itself over many years, atherosclerosis formation may cumulate into an acute burst of symptomatic arterial thrombus formation.

In the venous system, thrombus formation results from a decrease in blood flow, in conjunction with a hypercoagulable state and endothelial dysfunction (Virchow's triad), and most often affects the deep veins of the legs (deep vein thrombosis, DVT). The most serious complication of DVT is the embolisation of clot dislodgements to the lungs (pulmonary embolism, PE).

Numerous epidemiological studies report consistent associations between exposure to urban air pollution and cardio-respiratory morbidity and mortality. One of the important discoveries of these epidemiological studies during the last decade was that the increased mortality associated with enhanced air pollution exposure was not due only to pulmonary diseases, but mainly to *cardiovascular* diseases. (3-13).

The focus in the initial epidemiological research was directed towards the association between both short-term and long-term exposure to air pollution and arterial cardiovascular effects, such as myocardial infarction. These landmark studies, in the beginning of the 90's, were quickly followed by experimental studies in humans and in rodents, to unravel the underlying pathophysiological mechanisms. The number of publications in this field increased exponentially, so that in the middle of 2011, a search through PubMed using the MeSH terms 'air pollution' and 'cardiovascular disease' retrieved almost 1400 hits.

Active cigarette smoking has been established as a major independent cause of cardiovascular disease (14). The inhaled dose of fine particles from ambient air pollution, as from secondhand cigarette smoke, is extremely small compared with that from active cigarette smoking. Accordingly, the estimated relative risks from active smoking, even at relatively light smoking levels, are substantially larger than the risks from ambient air pollution or secondhand smoke. However, the risks induced by these latter 2 types of exposure are higher than would be expected from a simple linear extrapolation based on the amount of inhaled PM from active smoking (15), and have important public health implications (16).

Arterial and venous thrombosis share common risk factors (17). The role of air pollution exposure as a risk factor for *arterial* events now being beyond discussion, a few years ago, epidemiologists started investigating a possible association with *venous* thrombotic events. Thus, in 2008, Baccarelli et al. demonstrated a link between chronic exposure to elevated levels of air pollution and deep vein thrombosis (DVT) for the first time (18).

The following paragraphs will describe how air pollutants affect arterial and venous functionality and lead to pathophysiological manifestations.

Particle triggered pathophysiological mechanisms

Inhaled particles deposit in various segments of the human respiratory tract. While the larger PM_{10} particles impact to a large extent in the nasopharyngeal and tracheal region, the smaller $PM_{2.5}$ particles penetrate deeper into the bronchi and bronchioli, whereas the UFP reach the alveolar regions. Inhaled particles are believed to affect the cardiovascular system through 3 different pathways: interference with the autonomic nervous system, direct translocation of UFP into the systemic circulation and pulmonary inflammation.

PM inhalation deranges the autonomic nervous control of the heart (1). Numerous studies (e.g. (19, 20)) have shown that, by reducing the heart rate variability, PM may increase the risk for cardiac arrhytmias and sudden death. In addition, elevations in air pollution have been associated with ST-segment depression (21, 22), an impaired cardiac deceleration capacity (23), hypertension (24) and increased diastolic blood

pressure (25). The exact underlying mechanisms remain to be elucidated, but stimulation of irritant receptors in the airways and subsequent reflex activation of the nervous system as well as direct effects of pollutants on cardiac ion channels have been suggested (1, 26).

A second mechanism of action comprises the translocation of inhaled particles into the systemic circulation. Direct effects may occur via UFP that readily cross the pulmonary epithelial barrier, along with soluble constituents released from the larger particles (e.g. transition metals). Systemic translocation of particles was demonstrated in experimental animal models (27) (28). Although evidence of systemic translocation from human studies is less clear, with both positive (29, 30) and negative (31) findings, it is likely that this pathway also exists in humans, given the deep penetration of UFP into the alveoli and the close apposition of the alveolar wall and the capillary network. Radioactivity in the systemic circulation was already detected 1 minute after the inhalation of radioactively labelled carbon particles in humans, with peak radioactivity levels between 10 and 20 minutes (29). When measured in rats under resting conditions, only a small fraction (1.6-2.5%) of intratracheally instilled UFP translocated into the circulation. However, this fraction increased to 4.7% following pretreatment of the lungs with lipopolysaccharides, suggesting a role for pulmonary inflammation in enhancing the extrapulmonary translocation of particles (32). Different translocation mechanisms, ranging from endocytosis by alveolar type I and endothelial cells, over phagocytosis by macrophages to passage through widened tight junctions are recognized and depend on the particle surface chemistry (33). Once UFP have translocated to the blood circulation, they can be distributed throughout the body, and interact with the vascular endothelium or circulating cells, such as blood platelets and leukocytes.

Inhaled PM executes its deleterious effects also via a third, more chronic mechanism, namely pulmonary inflammation and oxidative stress. Exposure to PM induces a proinflammatory response in human lungs (34), consistent with observations in *in vivo* animal models (35, 36) and *in vitro* cellular models (37, 38). The presence of soluble transition metals in PM enhances the inflammatory responses via increased oxidative stress (39). The PM-induced pulmonary inflammation is followed by the release of inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and granulocyte

macrophage colony-stimulating factor (40) in the circulation, resulting in the release of bone-marrow derived neutrophils and monocytes (41).
The generation of a systemic inflammatory response, mostly demonstrated by increases in C-reactive protein (CRP) (42, 43), is of major importance in the pathogenesis of cardiovascular events. Upon PM exposure, IL-6 translocates from the lung into the systemic circulation (44) and is directly involved in the regulation of the synthesis of CRP in the liver. Elevated concentrations of IL-6 are associated with an increased risk of cardiovascular events (45, 46) and mortality (47). Knock-out mice that lacked IL-6 were protected against the prothrombotic effects of PM exposure (48). Increasing evidence points to an extensive cross-talk between inflammation and hemostasis, whereby inflammation leads to activation of blood platelets and of coagulation, and activated blood platelets and coagulation factors also considerably contribute to the inflammatory action (49).

In the following paragraphs, the deleterious effects of PM exposure on endothelial function and arterial and venous parameters will be discussed. By virtue of their respective protagonist roles, blood platelet activation will mainly be discussed in the paragraph on arterial events, while coagulation activation will mainly be discussed in the paragraph on venous events. Formally, arterial thrombosis, the basis for myocardial infarction, is the result of vessel wall injury and formation of a platelet-rich thrombus. Venous thrombosis, the basis for VTE (venous thromboembolism) results from coagulation activation and formation of a fibrin-rich thrombus. It should be noted, however, that both blood platelet and coagulation activation intervene in arterial and venous thrombosis, and that both systems highly interact with each other (50).

Endothelial function and fibrinolysis

The effects of air pollution on the endothelial function and the fibrinolytic system have mainly been investigated in controlled exposure studies by 2 research groups who joined forces. The groups of Newby and Blomberg used exposure chambers to expose healthy and compromised volunteers to the diluted exhaust of an iddling diesel engine for several hours in randomized cross-over studies. They demonstrated an impaired bradykinin-induced endothelial release of tissue plasminogen activator (t-PA) upon diesel exhaust inhalation (estimated reduction of net t-PA release of 34%)

(22, 51), in addition to an attenuated agonist-induced increase in blood flow at 6 hours post-inhalation, in the absence of inflammatory changes (51). At 24 hours post-inhalation, endothelium-dependent vasodilation (induced by acetylcholine and bradykinin) remained impaired, while endothelium-independent vasodilation (using sodium nitroprusside and verapamil) and t-PA release were unaffected, in the presence of mild sytemic inflammation (52).

These and other (53-56) studies did not demonstrate an assocation between PM exposure and baseline levels (not bradykinin-induced) of t-PA.

While studies, based on controlled exposure to diluted diesel exhaust (22, 52, 57) or concentrated ambient particles (55), did not observe increases in the levels of plasminogen activator inhibitor-1 (PAI-1), some epidemiological or animal studies, focussing on urban PM, did: a study in 76 young healthy students demonstrated elevated PAI-1 concentrations in association with the mean $PM_{2.5}$ or PM_{10} concentration at their university's campus over 1 to 3 days (54). Likewise, urban PM upregulated PAI-1 levels, 24 hours after intratracheal instillation in mice (58).

PM exposure could also impair the endothelial repair mechanisms by reducing the number of endothelial progenitor cells, as demonstrated by a recent report (59).

Taken together, these studies indicate a potential deleterious effect of PM inhalation on the endothelial and fibrinolytic function that may aggravate the prothrombotic phenotype induced by blood platelet and coagulation activation.

Air pollution and arterial events

Over the last 2 decades, a vast number of epidemiological studies (reviewed in (60)) have provided convincing evidence to conclude that chronic exposure to PM enhances atherosclerosis and that acute exposure increases the thrombotic risk associated with atherosclerotic plaque rupture, triggering arterial thrombosis, myocardial infarction and cardiovascular mortality. Relative risk levels for cardiovascular disease may vary between different studies, due to differences in study design. Short-term effects have been most often studied in time-series and case-crossover studies, while long-term effects have been studied in case-control and cohort studies. Relative risk levels are generally lower in time series studies than in other epidemiological designs. Nevertheless, the associations between cardiovascular disease and PM exposure are consistent, whatever the type of method used (60).

An initial landmark report was that of the Harvard Six Cities study (5), in which a cohort of 8111 adults were followed up for 14 to 16 years. The adjusted overall mortality rate for the most polluted city vs. the least polluted was 1.26 (95%CI 1.08-1.47). Cardiovascular deaths accounted for the largest single category of increased mortality. Each 10 $\mu g/m^3$ increase in long-term levels of $PM_{2.5}$ has been associated with a 8 to 18% increase in cardiovascular mortality (7). An association with mortality was also found for traffic-related air pollution and several traffic exposure variables, although relative risks were small (61). The effects of long-term PM exposure on cardiovascular mortality have been shown elegantly by the demonstration of a parallelism between air quality improvement and reduction in cardiovascular events on a population-based level (62, 63). A potential benefit in general mortality can be expected within 2 years after the reduction of PM exposure (64).

The magnitude of these associations appeared to be more pronounced for the smaller $PM_{2.5}$ fraction than for the larger PM_{10} fraction (65). Considering a large body of evidence, a recent updated version of the American Heart Association scientific statement on 'Air Pollution and Cardiovascular Disease' (66) concluded that per 10 $\mu g/m^3$ increase in long-term levels of $PM_{2.5}$, all-cause mortality increased by an approximate 10%. The mortality risk specifically related to cardiovascular disease appears to be elevated to a similar, or perhaps even greater extent, ranging from 3 to 76% over different studies.

Chronic PM exposure and atherosclerosis

What etiological agent can explain the link between chronic air pollution exposure and cardiovascular mortality? Künzli et al. provided the first epidemiological evidence for an association with atherosclerosis: living in the areas of Los Angeles with highest annual mean concentrations of ambient $PM_{2.5}$ was associated with an increased intima-media thickness of the carotid artery (67).

Distance from the residence to a major road correlated with the degree of coronary artery calcification, a measure for atherosclerosis (68).

Another study in 5172 adults investigated 20-year PM exposure and found an association, although weaker than in the previous studies, with carotid intima media

thickness, but not with other measures of atherosclerosis i.e. coronary calcium and ankle brachial index (69).
A recent study demonstrates that long-term PM exposure is not only related to the degree, but also to a faster progression rate of atherosclerosis (70).

Along with this epidemiological evidence, experimental research also established a link between exposure to PM and the development of atherosclerosis. Repeated exposure to PM_{10} in rabbits was associated with both systemic inflammation and the progression of the atherosclerotic process, the extent of which correlated with the extent of PM_{10} phagocytosed by alveolar macrophages (71).
Exposing genetically susceptible apolipoprotein E-null mice for 6 months to an equivalent concentration of 15.2 $\mu g/m^3$ $PM_{2.5}$ over a lifetime, enhanced abdominal aortic plaque formation as compared to mice exposed to filtered air (72). Interestingly, ultrafine (<0.18 μm) particle-exposed mice exhibited significantly larger atheroslerotic lesions than mice exposed to fine (<2.5 μm) particles or filtered air (73).

Atherosclerosis is now considered an inflammatory disease with low density lipoprotein (LDL) cholesterol accumulation in the arteries as the primary risk factor (2). However, up to 50% of the patients who develop atherosclerosis do not have high cholesterol (74). Therefore, it is the relationship between the accumulated lipids and other harmful components of inflammation in the arterial vessel wall that is of concern. LDL infiltration of the arterial vessel wall is followed by oxidative modification to oxidized LDL (ox-LDL) in the subendothelial space and chemotaxis of monocytes. These monocytes differentiate into macrophages and the subsequent phagocytosis of ox-LDL leads to the formation of foam cells and the release of inflammatory mediators, inducing a vicious cycle of inflammation. Further stages include smooth muscle cell proliferation, formation of a fibrous cap with necrotic core and calcification (2). Thickening of the vessel wall and obliteration of the vascular lumen induces downstream ischemia of the tissues.

PM exposure can induce atherosclerosis via different pathways: systemically translocated UFP or their chemical constituents induce activation of proatherogenic

molecular pathways in endothelial cells, by oxidative stress. Inflammatory mediators released from the lungs may promote chemotaxis of monocytes into the vessel wall. PM induces high-density lipoprotein (HDL) dysfunction with loss of its anti-inflammatory properties (75).

Oxidative transformation of LDL into ox-LDL is a key step in the initiation and progression of atherosclerosis (76), and circulating levels of ox-LDL are therefore an early marker, and a risk factor for the disease (77). The correlation between PM exposure and circulating levels of ox-LDL on an individual level was shown by Jacobs et al., demonstrating a dose-dependent association between this parameter and the carbon load of airway macrophages, a personal marker for chronic exposure to fossil fuel derived ultrafine particles (78).
It has been previously shown that particles can induce oxidative stress both *in vitro* (79, 80) and in exposed animals (73, 81-83).
In agreement with epidemiological findings (65), experimental studies suggest that the smaller particles are more pathogenic, as a result of their greater propensity to induce systemic prooxidant and proinflammatory effects (73). Indeed, ambient UFP trigger the induction of the antioxidant gene heme oxygenase 1 (HO-1) to a higher degree than ambient $PM_{2.5}$ or coarse particles, both *in vitro* (84) and *in vivo* (73, 75).
Several mechanisms contribute to the greater proatherogenic potential of UFP: because of their small size, particles <0.1-0.2 μm contribute very little to overall $PM_{2.5}$ mass. However, they represent >85-90% of the total $PM_{2.5}$ particle number (85). The high number of UFP, in conjunction with a large surface-to-mass ratio increases the bioavailability of the pro-oxidant chemicals (polycyclic aromatic hydrocarbons, transition metals etc.) present on the UFP's surface. The number of chemicals that are displayed on the surface of particles increases exponentially as the size shrinks below 100 nm (33). Deep penetration in the lung and subsequent translocation of UFP into the circulation make these pro-oxidant chemicals more bioavailable at the contact sites of the particles with cells and tissues.

Acute PM exposure and arterial thrombosis

Not only chronic, but also short-term PM exposure has been linked to cardiovascular mortality: Both the American NMMAPS (National Morbidity, Mortality, and Air Pollution Study (13)) and the European APHEA2 (Air Pollution and Health: A

European Approach (3, 12)) studies (approximately 50 million and 43 million persons included respectively) demonstrated small increases in cardiovascular mortality with increasing PM exposure. In an attempt to evaluate the coherence of studies across continents, the APHENA (A Combined European and North American Approach) analyzed data of these 2 aforementioned studies and Canadian studies (86). The combined effect on all-cause mortality ranged from 0.2% to 0.6% for a 10 $\mu g/m^3$ increase in daily levels of ambient PM_{10}, with greater effects for the elderly (>75 years) and the unemployed. An extensive review of studies investigating a link between short-term PM exposure and cardiovascular mortality is provided in (66).

Peters et al. (87) demonstrated an increased risk of myocardial infarction in association with elevated concentrations of fine $PM_{2.5}$, both in the previous 2-hours period and the day before the onset. Likewise, the onset of myocardial infarction was linked to participation in traffic, as soon as 1 h afterward (odds ratio 2.92, 95%CI 2.22-3.83) (88).

Exposure to ambient $PM_{2.5}$ is associated with short-term increases in hospital admission rates for cerebrovascular, peripheral and cardiac ischemic disease, heart rhythm and heart failure, with the strongest association for heart failure (1.28 % 95%CI 0.78-1.78% increase in risk per 10 $\mu g/m^3$ increase in same-day $PM_{2.5}$) (89).

The risk of mortality from coronary heart disease related to PM exposure appears to be higher in women (RR 1.42, 95%CI 1.06-1.90) than in men (RR 0.90, 95%CI 0.76-1.05 per 10 $\mu g/m^3$ increase in $PM_{2.5}$)(90). In a study of 65,893 postmenopausal women with a median follow-up of 6 years, each increase in long-term levels of $PM_{2.5}$ of 10 $\mu g/m^3$, measured at the women's residence, was associated with a 24% (95%CI 09-41%) increase in the risk of a cardiovascular event, and a 76% (95%CI 25-147%) increase in the risk of death of cardiovascular disease (91).

Although the magnitude of the risk on myocardial infarction induced by short-term PM exposure is rather small on a personal level, it is of major importance on a population level, by virtue of the large number of people exposed. Taking into account both risk magnitude and risk prevalence by measurement of the population attributable fraction (PAF), Nawrot et al. showed that a short-term increase in air pollution exposure is an important trigger for myocardial infarction, of similar magnitude (PAF 5-7%) as other well accepted triggers such as physical exertion, alcohol and coffee (16).

Epidemiological studies suggest an association between short-term increases in PM exposure and atherosclerotic plaque rupture, causing arterial thrombosis and myocardial infarction. In contrast to the growing number of mechanistic studies investigating the role of chronic PM exposure on atherogenesis, the precise mechanisms explaining the role of short-term PM exposure in acute plaque rupture largely remain to be elucidated. However, several epidemiological and mechanistic studies demonstrated that, in parallel to atherosclerotic plaque rupture, direct or indirect activation of circulating blood platelets by PM contributes to the arterial thrombosis risk. Indeed, the extent to which a growing thrombus occludes the vascular lumen may in part depend on platelet hyperactivity.

Under physiological circumstances, the high blood pressure generated on the arterial side of the circulation requires a powerful, almost instantaneous prohemostatic response in order to minimize blood loss from sites of vascular injury. Blood platelets play a critical role in this response. Upon damage of the endothelial cell layer covering the luminal side of blood vessels, circulating blood platelets adhere to the exposed subendothelial matrix through the binding of the glycoprotein (GP) Ib-IX-V receptor to exposed von Willebrand factor (VWF). Blood platelet adhesion is further enhanced by the binding of different GP receptors to other subendothelial matrix proteins, such as collagen and fibrin(ogen). Upon adhesion and activation of the blood platelets by various agonists, VWF and fibrinogen molecules cross-link different platelets, resulting in blood platelet aggregation and the formation of an initial platelet plug which covers the site of endothelial lesion. The simultaneous activation of the coagulation cascade leads to the formation of a network of insoluble fibrin strands that further stabilize the initial platelet plug.

Air pollution exposure can induce an inappropriate activation of blood platelets beyond the physiological need to restore vessel damage, resulting in arterial thrombosis (Fig. 2).

By exposing healthy volunteers to diluted diesel exhaust, Lucking et al. showed an association with enhanced platelet activity and thrombus formation in an *ex vivo* perfusion chamber, 2 hours and 6 hours after exposure, in conjunction with increased numbers of platelet-neutrophil (+52%) and platelet-monocyte (+30%) conjugates (92).

Short-term, but not long-term PM exposure was found to enhance platelet function, as measured *ex vivo* by a shortening of the closure time of the Platelet Function Analyzer (PFA-100, Siemens Healthcare Diagnostics), in patients with diabetes (93). In this study, an interquartile range (39.2 μg/m^3) increase in the PM_{10} concentration, measured 2 hours before the clinical investigation at the entrance of the hospital, was associated with a decrease of 21.1 sec (95%CI -35.3 to -6.8) in the PFA-100 closure time. Platelet function was not correlated with leukocyte counts, suggesting that short-term PM exposure may have effects on platelet function independently of systemic inflammation, as was also shown in experimental animal models (35).

Ambient PM_{10} levels have also been associated with augmented platelet aggregation 24 to 96 hours after exposure in healthy adults, in the absence of increased CRP or fibrinogen (94). In patients with coronary heart disease, mean concentrations over 24 hours of ambient UFP, but not $PM_{2.5}$ or PM_{10} were positively associated with the levels of soluble CD40 ligand, a marker for platelet activation. No assocations were found with longer time frames, up to 5 days (95).

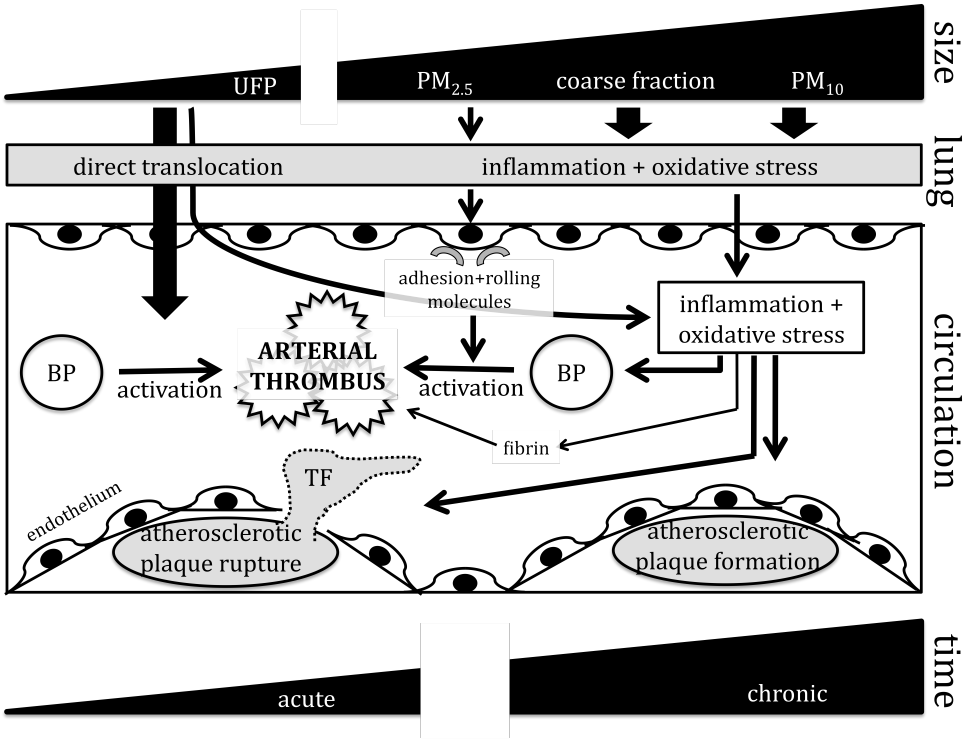

Figure 2: Biological pathways linking PM exposure and arterial thrombosis
BP: blood platelet, PM: particulate matter, TF: tissue factor, UFP: ultra-fine particles

In experimental conditions using DEP, Nemmar et al. demonstrated a prothrombotic tendency and activation of circulating blood platelets (confirmed by PFA-100), as well as lung inflammation, which persisted up to 24 hr after intratracheal instillation of DEP in hamsters (35, 96).

However, different pathophysiological mechanisms seem to be responsible for the observed prothrombotic risk at different time points. Pretreatment of hamsters with a histamine H1-receptor antagonist, an anti-inflammatory drug, abolished pulmonary inflammation at all time points and reduced DEP-induced thrombosis at 6 and 24 hours post-instillation, indicating a crucial role for inflammation in thrombogenicity at these time points. Likewise, the administration of other anti-inflammatory drugs, such as dexamethasone and selective inhibitors of basophils, macrophages and neutrophils, also significantly reduced the PM-induced prothrombogenicity at 24 hours (97, 98). In contrast, pretreatment with the histamine H1-receptor antagonist did not reduce thrombosis as soon as 1 hour after DEP exposure (35).

Therefore, the early prothrombotic tendency appears not to result from pulmonary inflammation, but possibly from direct effects of systemically translocated particles on the blood platelets and/or the (pulmonary) vessel wall (35). The direct activating effect of PM on blood platelets was shown by the addition of as little as 0.5 μg/mL DEPs to untreated hamster blood, significantly shortenening the PAF-100 closure time (96), as well as by a dose-dependent (0.1-1 μg/mL) effect of PM on *in vitro* platelet aggregation in rat blood (99), although similar experiments in human blood were negative (94).

In agreement with these results, 1 hour after intratracheal instillation, well-defined positively charged ultrafine (60 nm) polystyrene particles significantly enhanced platelet-rich thrombus formation, while 400 nm particles, incapable of systemic translocation, did not affect thrombus formation, despite similar increases in neutrophils, lactate dehydrogenase and histamine levels in the bronchoalveolar lavage fluid (100).

Pulmonary instillation of carbon nanotubes elevated platelet-leukocyte conjugates at 6 hours and increased the peripheral thrombotic potential at 24 hours after exposure. Inhibition of P-selectin abrogated these responses (101). P-selectin is found in storage Weibel-Palade bodies of endothelial cells and in α-granules of platelets, from where it can be expressed on the outer membrane upon activation. Surface expression of P-

selectin initiates capture and rolling of circulating leukocytes over stimulated endothelium (102) and the formation of platelet-leukocyte conjugates (103). Increased levels of platelet-leukocyte conjugates have been demonstrated in Indian women who used biomass as cooking fuel, producing higher levels of PM, as compared to women cooking with a cleaner fuel (liquefied petroleum gas) (104). In a panel study of 60 elderly subjects with coronary artery disease, Delfino et al. demonstrated associations between soluble P-selectin levels and the mean 1 to 5-day concentrations of ambient finer particles ($PM_{0.25}$ and $PM_{2.5}$), but not the bigger PM_{10} (105). Taken together, these studies suggest that the release of pulmonary cell-derived mediators (eg. histamine) and the expression of endothelial and platelet surface proteins (eg. P-selectin) after several hours, along with the more rapid activation of circulating platelets by direct contact with UFP may mediate peripheral prothrombotic effects.

Air pollution and venous thromboembolism

Epidemiology

In addition to the well-recognized PM-related adverse effects on the arterial vascular system, recent epidemiological evidence also suggests an association between exposure to PM and venous thromboembolism (VTE). Baccarelli et al. reported a 70% increase in the risk of deep vein thrombosis (DVT) for each 10 $\mu g/m^3$ increase of the annual mean level of PM_{10} in the areas of residence of the study subjects (OR 1.70, 95%CI 1.30-2.23) (18). The observed exposure-response relationship was approximately linear over the observed PM_{10} range, so that PM_{10} at the higher concentrations within the international limits can still increase the risk of DVT, as compared to the lowest concentration measured. These authors found, in the same study subjects, that living near major traffic roads was also associated with an increased risk of DVT, even after controlling for the community-level PM pollution (106). Very recently, exposure to PM has also been associated with hospital admission for VTE in Chile. Both for DVT and for PE, pooled estimates of relative risk of hospitalization were 1.05 (95%CI 1.03-1.06) for a 20.02 $\mu g/m^3$ increase in $PM_{2.5}$ (107).

Table 1: Associations between PM exposure and coagulatory changes according to different studies

reference			exposure			coagulatory changes	
author	**year**	**type**	**controlled exposure**	**type of air pollution**	**exposure time**	**significant changes**	**no significant changes**
Seaton (108)	1999	NA	no	ambient PM_{10}	3 days	FVII (-), fbg (-)	
Ghio (34)	2000	healthy subjects	yes	concentrated $PM_{2.5}$	2h	fbg	
Pekkanen (109)	2000	healthy subjects	no	ambient PM_{10}	1-3 days	fbg	
Ghio (55)	2003	healthy subjects	yes	concentrated $PM_{2.5}$	2h	fbg	D-dim, PC, VWF
Riediker (110)	2004	healthy subjects	no	in-vehicle $PM_{2.5}$	9h	VWF	
Becket (111)	2005	healthy subjects	yes	zinc oxide particles	2h		FVII, fbg, VWF
Blomberg (112)	2005	COPD patients	yes	diesel exhaust	1h		fbg, D-dim, VWF
Barregard (113)	2006	healthy subjects	yes	wood smoke	4h	FVIII	fbg, FVII, D-dim, VWF
Ruckerl (114)	2006	CHD patients	no	ambient $PM_{2.5}$ and PM_{10}	1-5 days	FVII (-), VWF	fbg, D-dim
Baccarelli (115)	2007	healthy subjects	no	ambient PM_{10}	t0 - 30days	PT	aPTT, fbg, AT, PC, PS
Carlsten (57)	2007	healthy subjects	yes	diesel exhaust	2h		D-dim, VWF
Chuang (54)	2007	healthy students	no	ambient $PM_{2.5}$ and PM_{10}	1-3 days	fbg	
Ruckerl (116)	2007	MI survivors	no	ambient $PM_{2.5}$ and PM_{10}	1-4 days	fbg	
Scharrer (117)	2007	healthy subjects	yes	welding fume	1h		FVIII, VWF, AT
Brauner (118)	2008	healthy subjects	yes	indoor $PM_{2.5}$ and PM_{10}	2 days		fbg, FII+VII+X
Lucking (92)	2008	healthy subjects	yes	diesel exhaust	1-2h		PT, aPTT
Rudez (94)	2009	healthy subjects	no	ambient PM_{10}	6h - 4days		fbg,TG
Samet (56)	2009	healthy subjects	yes	concentrated ambient UFP	2h	D-dim	fbg, FIX, FXII, VWF
Bonzini (53)	2010	steel plant workers	no	occupational PM_{10}	1-3 days	PT,TG	aPTT, D-dim
Stewart (119)	2010	T2DM patients	yes	carbon UFP	2h		FVII, FIX, D-dim, TF
Thompson (120)	2010	healthy subjects	no	ambient $PM_{2.5}$	t0 - 7 days		fbg
Jacobs (78)	2011	DM patients	no	carbon load in alveolar Mφ	NA		VWF

COPD: chronic obstructive inflammatory disease, MI: myocardial infarction, CHD: coronary health disease, DM: diabetes mellitus, T2DM: type 2 diabetes mellitus, PM: particulate matter, UFP: ultra-fine particles, Mφ: macrophages, PT: prothrombin time, aPTT: activated partial prothrombin time, AT: antithrombin, PC: protein C, PS: protein S, F: factor, fbg: fibrinogen, D-dim: D-dimers, VWF: von Willebrand factor, TG: thrombin generation, TF: tissue factor. (-) denotes inverse correlations. Values between brackets denote non-significant trends. NA: data not available

However, these initial epidemiological reports on the association between air pollution exposure and venous thrombosis were followed by a number of prospective cohort studies that failed to demonstrate an association (121, 122) (see general discussion).
Hence, in contrast to the well-accepted and documented deleterious effects of air pollution exposure on arterial events, data are scarce and the link with venous thrombosis is less straightforward, prompting further epidemiological investigation.

Pathophysiology
At lower rates of shear found in the venous circulation, the contribution of blood platelets to clot formation is of lesser importance than in the arterial circulation, leaving a protagonist role for the coagulation cascade in venous hemostasis. Activation of the coagulation cascade is initiated by activation of coagulation factor VII (FVII) by binding to tissue factor (TF), expressed on subendothelial cells such as fibroblasts and vascular smooth muscle cells. The complex of TF and activated FVII (FVIIa) initiates a cascade of subsequent coagulation factor activations, resulting in the generation of thrombin. Thrombin (FII) is a key enzyme, converting fibrinogen monomers to fibrin polymers that clot into a fibrin plug, and amplifying the coagulation cascade through activation of FV, FVIII and FXI.
The mechanisms underlying the observed increase in venous thrombosis in association with exposure to air pollution remain largely unknown, and published results with regard to markers of secondary hemostasis activation are conflicting. Although some epidemiological and controlled exposure studies demonstrated an association between PM exposure and shortening of the prothrombin time (PT) or increased levels of fibrinogen and VWF, others failed to demonstrate positive associations with these or other markers of coagulation, in humans (Table 1). In fact, disappointingly few studies reported on PM-induced coagulatory changes that could form the basis for the observed link between air pollution and DVT.

VTE is a common disease, and aging raises the relative risk for DVT from about 1/10,000 at the age of 40 to 1/100 at the age of 80 (123). In view of the combination of the comonness of air pollution exposure and an ageing population in the western world, research into the association between air pollution exposure and VTE has substantial public health relevance.

It was the conundrum of air pollution-associated risk of venous thrombosis in the absence of well-documented changes in secondary hemostasis parameters that formed the basis for the present thesis.

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OBJECTIVES

Development of a mouse model of PM-induced venous prothrombogenicity and identification of pathophysiological mechanisms.

The short-term effects of PM exposure on primary hemostasis activation are well known. In contrast, whether short-term PM exposure also induces changes in secondary hemostasis parameters, or whether a more sustained exposure time is warranted, remained to be elucidated. Epidemiological studies show that the increased risk of VTE is associated with long-term, but not with short-term exposure to PM (1). Therefore, we aimed to identify changes in both inflammatory and prothrombotic parameters upon short-term and long-term exposure to air pollution.

Acute and subacute exposure of mice to PM by intratracheal instillation

Mice were exposed to 2 types of PM via a single ('acute') or 3 repetitive ('subacute') intratracheal instillations. Besides the analysis of parameters of inflammation and coagulation *ex vivo*, thrombosis experiments were also performed *in vivo*. (Chapter 1)

Subchronic exposure of mice to PM

Mice were subchronically exposed to traffic-related air pollution by placing them in a roadside tunnel for several weeks. (Chapter 4)

Epidemiological analysis of the associations between air pollution exposure and increased risk factors for VTE

To unravel the pathophysiological mechanisms underlying the described association between PM exposure and VTE in humans, we assessed several parameters of inflammation and coagulation in a group of patients with diabetes, and correlated the results with different measures of both recent and chronic exposure to air pollution. (Chapter 2)

Interaction between risk factors

Epidemiological research indicates that some populations are more susceptible to the deleterious health effects of PM exposure. Several studies suggest that susceptible individuals may, amongst others, include the elderly (2, 3) and patients with diabetes (4-6). The aforementioned epidemiological study was performed in a group of susceptible patients with diabetes (Chapter 2).

We also investigated to which extent aging affects the PM-induced procoagulant changes in mice subchronically exposed to PM (Chapter 4). Baseline (i.e. unexposed) differences in coagulation parameters and microvesicles (see below) between elderly and young persons were investigated in Chapter 3.

Role of microvesicles

Microvesicles, cell-derived vesicles with a diameter <1 μm, are believed to play a central role in the interface between inflammation and hemostasis. Some publications indirectly suggest a role for microvesicles in PM-induced prothrombotic changes (7, 8), but neither the demonstration of a direct association, neither the identification of the pathological fraction of microvesicles in this regard has been achieved yet. By using both antigenic and functional assays, we aim to clarify the role of microvesicles in our animal model of chronic PM inhalation (Chapter 4), as well as in the human epidemiological study (Chapter 2).

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CHAPTER 1

Short-term Exposure to Particulate Matter induces Arterial but not Venous Thrombosis in healthy Mice

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Abstract

Background: Epidemiological findings suggest an association between exposure to particulate matter (PM) and venous thrombo-embolism.

Objectives: To investigate arterial vs venous thrombosis, inflammation and coagulation in mice, (sub)acutely exposed to 2 types of PM.

Methods: Various doses (25, 100, 200 μg/animal) of urban particulate matter (UPM) or diesel exhaust particles (DEP) were intratracheally instilled in C57Bl6/n mice and several endpoints measured at 4, 10 and 24 hours. Mice were also repeatedly exposed to 100 μg/animal on 3 consecutive days with endpoints measured 24 hours after the last instillation.

Results: Exposure to 200 μg/mouse UPM enhanced arterial thrombosis, but neither UPM nor DEP significantly enhanced venous thrombosis. Both types of PM induced dose-dependent increases in broncho-alveolar lavage fluid (BALF) total cell numbers (mainly neutrophils) and cytokines (IL-6, KC, MCP-1, RANTES, MIP-1α), with peaks at 4 hours and overall higher values for UPM than for DEP. Systemic inflammation was limited to increased serum IL-6 levels, 4 hours after UPM. Both types of PM induced similar and dose-dependent but modest increases in FVII, FVIII and fibrinogen. Three repeated instillations did not or only modestly enhance the proinflammatory and procoagulant status.

Conclusions: Compared to DEP, UPM induced more pronounced pulmonary inflammation, but both particle types triggered similar and mild short-term systemic effects. Hence, acute exposure to PM triggers activation of primary haemostasis in the mouse, but no substantial secondary haemostasis activation, resulting in arterial but not venous thrombogenicity.

Introduction

Numerous epidemiological studies reported consistent associations between exposure to urban air pollution, especially by particulates, and cardio-respiratory morbidity and mortality (1-4). Over the last 2 decades, a vast number of both epidemiological and mechanistic studies have provided convincing evidence to conclude that chronic exposure to particulate matter (PM) enhances atherosclerosis and that acute exposure increases the risk of atherosclerotic plaque rupture and blood platelet activation, triggering arterial thrombosis and myocardial infarction (5-9).

In addition to the well-recognized PM-related adverse effects on the arterial vascular system, recent epidemiological evidence also suggests an association between exposure to PM and venous thrombo-embolism (VTE). Baccarelli et al. reported a 70% increase in the risk of deep vein thrombosis (DVT) for each 10 $\mu g/m^3$ increase of the annual mean level of PM with aerodynamic diameter $\leq$10 μm (PM_{10}) in the areas of residence of the study subjects (10). These authors found, in the same study subjects, that living near major traffic roads was also associated with an increased risk of DVT, even after controlling for the community-level PM pollution (11). Very recently, exposure to PM has also been associated with hospital admission for VTE in Chile (12). The mechanisms underlying the observed increase in venous thrombosis remain largely unknown, and published results with regard to markers of secondary haemostasis activation are conflicting. Although some epidemiological and controlled exposure studies demonstrated an association between PM exposure and shortening of the prothrombin time (PT) (13, 14) and increased levels of fibrinogen (15-18), von Willebrand factor (19, 20) or plasminogen activator inhibitor-1 (PAI-1) (16), others failed to demonstrate any association with these markers (21-25). Also in the limited number of experimental studies in rodents, investigating the PM-induced changes in secondary haemostasis, results are conflicting (26-29). This discrepancy in different studies is likely to be attributed to differences in experimental design and in the composition of PM. Among other factors, animal studies differ in dosing of PM, the type of exposure, lag time between PM exposure and sampling, and the type of PM animals are exposed to. Both urban particulate matter (UPM) (26, 28, 29) and diesel exhaust particles (DEP) (27) are frequently used.

In an attempt to take into account the determining role of variable exposure parameters, we investigated how and whether standardized short-term experimental exposure of mice to 2 different types of PM would affect experimental venous thrombosis and activate the coagulation system, the activation of which is central in VTE. Different time frames and doses were applied.

Materials and methods

Particle collection

UPMs with a mean aerodynamic diameter smaller than 10 μm (PM_{10}) were collected as previously described (30) using a high volume sampler (GMW Model 1200, VFC HVPM10; Sierra Andersen, Smyrna, GA, USA) in the industrial region of the Metropolitan Zone of Mexico City, in Xalostoc from November 2004 till April 2005 (Mexican Consortium for Particulate Matter Studies). Elemental composition is shown in table E1 (data supplement).

DEPs were generated using a 4-cylinder Deutz BF4M1008 diesel engine and collected in a baghouse at the US EPA's National Risk Management Research laboratory (RTP, NC) from October till November 2004 (kind gift of Dr A Farraj, National Health and Environmental Effects Research Laboratory, NHEERL) (31).

LPS content of both types of PM was measured as described previously (32) and found to be 138.4 endotoxin units (EU)/mg for UPM and smaller than 0.05 EU/mg (detection limit) for DEP.

Intratracheal instillation of particles

This study was approved by the Institutional Review Board of the University of Leuven, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Particles were suspended in vehicle (NaCl 0.9%, containing 0.1% Tween 80). To minimize aggregation, particle suspensions were always sonicated for 15 minutes and vortexed immediately (<1 minute) before their IT instillation in 8- to 10-week old male C57Bl6/n mice, previously anesthetized with sodium pentobarbital (60 mg/kg). A Becton Dickinson 22 Gauge cannula was inserted via the mouth into the trachea and 50 μL of PM suspension was instilled via a syringe, followed by an air bolus of 100 μL. Controls received vehicle only.

The IT instillation of a bolus of particles could be considered as non-physiologic, but this method of delivery has been shown to be a convenient and valid, although admittedly not

perfect, mode of administration of foreign compounds into the airways (33) and has been used in numerous PM-based studies (7, 26, 27, 29).

Experimental design

Arterial and venous thrombotic occlusion times were recorded 24 hours after a single IT instillation of vehicle or PM (200 μg/mouse). In each experimental group, n=5-7.

In dose-response experiments, samples were taken 24 hours after a single IT instillation of different doses of PM (25, 100 or 200 μg/mouse).

In time-dependence experiments, samples were taken at 4, 10 or 24 hours after a single ('acute') instillation of 100 μg of PM/mouse, or at 24 hours after the last of 3 consecutive ('subacute') daily instillations of 100 μg of PM/mouse.

Systemic coagulation parameters were measured at 24 hours after a single (different doses) or after repeated (100 μg doses) PM exposure. In each experimental group, n≥5.

Blood collection and analysis

Following anesthesia (sodium pentobarbital, 60 mg/kg, i.p.), blood was collected from the retroorbital sinus on citrate 0.38%. Blood cell counts and differentials were performed on a Cell-Dyn 3500R counter (Abbott, Diegem, Belgium). Samples were centrifuged twice (15,000 g x 10 min) and plasma was stored at -80°C for subsequent coagulation assays. Blood was also collected in a dry tube, incubated at 37°C for 1 hour, centrifuged (15,000 g x 10min) and serum was stored at -20°C for cytokine analysis.

Thrombotic occlusion time

Thrombotic occlusion times were measured as previously described (34). Platelets were fluorescently labelled *in vivo* by IV injection of Rhodamine 6G (3.3 mg/kg, Invitrogen, Merelbeke, Belgium). After opening the abdomen, mesenteric arteries and veins were exposed on the table of an inverted epifluorescent microscope (×10) (Nikon Diaphot 300), coupled to ImageJ software. Endothelial injury was induced by topical deposition of filter paper saturated with $FeCl_3$ (5%) (Sigma-Aldrich, Bornem, Belgium) for 3 minutes. Platelet deposition and thrombus growth were then monitored in real-time until complete occlusion occurred in the mesenteric artery and vein. The parameter analyzed was the vessel occlusion time, defined as an arrest of blood flow for at least 1 min. Mean values of up to two controlled lesions per animal are shown.

To investigate to which extent both occlusion times were determined by secondary haemostasis parameters, non-instilled mice were pretreated with 4 mg/kg low molecular weight heparin (LMWH: enoxaparin, Sanofi-Aventis, Diegem, Belgium) I.V. and arterial and venous occlusion times were compared to the values found in separate untreated controls.

Plasma assays

Activated partial thromboplastin time (aPTT) and prothrombin (PT) clotting times were determined on a semi-automated coagulometer (KC10, Amelung, Germany) using commercially available reagents (HemsIL Synthasil, IL, Zaventem, Belgium and Innovin, Siemens, Brussels, Belgium). Levels of the coagulation factors FVII and FVIII were measured in parallel, via a 1-stage clotting assay (FVII and FVIII Deficient Plasma, IL, Zaventem, Belgium), sensitive to *in-vivo* preactivation of clotting factors, and a chromogenic assay (Coaset FVII, Chromogenix, Milan, Italy and FVIII chromogenic assay, Siemens, Brussels, Belgium), insensitive to such preactivation. A fractionated murine plasma pool of different strains was used to make the calibration curves for the determination of FVII and FVIII. Fibrinogen levels were determined by commercial anti-murine fibrinogen ELISA (Gentaur, Brussels, Belgium).

Bronchoalvolar lavage fluid (BALF) collection and analysis

Following blood sampling, the abdomen was opened and mice were sacrificed by transsection of the abdominal vessels. The trachea was exposed, cannulated with a 20 Gauge cannula and lungs were lavaged three times with 0.7 mL (total volume of 2.1 mL) of sterile NaCl 0.9%. The recovered fluid aliquots were pooled and placed on ice. No difference in the volume of collected fluid was observed between the different groups. Cells in fresh BALF were stained with Trypan Blue and counted in a Bürker hemocytometer. Cell differentials were determined by light microscopy on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Siemens, Brussels, Belgium). The remaining BALF was then centrifuged (1000 g x 10 min.) and the supernatant stored at -20°C for cytokine analysis.

Cytokine analysis

We analyzed 6 cytokines involved in the activation of macrophages: interleukin-6 (IL-6), keratinocyte-derived chemokine (KC), monocyte chemoattractant protein-1 (MCP-1), RANTES, macrophage inflammatory protein-1a (MIP-1α) and interleukin-1b (IL-1β). Analysis was performed on both undiluted serum and BALF using a multiplex cytometric

bead assay for murine cytokines, purchased from Becton Dickinson (Erembodegem, Belgium). In separate experiments, the role of endotoxin (LPS) in pulmonary cytokine production was investigated in mice exposed to UPM, DEP, LPS or the combination of DEP and LPS (see data supplement).

Tissue collection and analysis

Lung and liver tissues were harvested, snap frozen in liquid nitrogen and stored at -80°C. RNA was extracted using chloroform extraction and isopropanol precipitation. After reverse transcription of RNA (1 μg) to cDNA, real-time PCR measurements of individual samples were performed in duplicate on the AB 7500 Fast PCR System (Applied Biosystems, Ottignies-Louvain-la-neuve, Belgium). FAM labeled primers were commercially obtained from Applied Biosystems (tissue factor: Mm00438853_m1; thrombomodulin: Mm00437014_s1; VCAM-1: Mm01320970_m1; ICAM-1: Mm00516023_m1; antithrombin: Mm00446573_m1; protein C: Mm00435966_m1; coagulation factor VII: Mm00487329_m1; fibrinogen beta-chain: Mm00805336_m1; GaPDH: Mm99999915_g1). GaPDH was used as house keeping gene for relative expression of C_t values and results are expressed as arbitrary units (AU).

Statistical analysis

All statistics were performed non-parametrically. Data are expressed as median with interquartile range. Mann-Whitney tests were used to analyze significance between thrombosis experiments in the presence or absence of LMWH. Statistical significance between groups at different time points after a single or repeated instillation of 100 μg PM or vehicle was analyzed by Kruskal-Wallis with Dunn's multiple comparison post-tests. Dose-response relationships were determined by Spearman correlation. This trend analysis was preferred over multigroup comparisons to limit the total number of statistical tests (multiple testing). This approach limits, but does not eliminate the possibility of a type-1 error. Therefore, we can not exclude that some of the significant (defined as $p<0.05$) results observed are chance findings. However, given the relatively small number of animals per group, a stringent correction would compromise the possibility of finding small changes in the parameters analyzed. All statistical analyses were performed using GraphPad Prism version 4.0b and Instat version 3 (GraphPad Software, San Diego, US).

Results

Pulmonary effects: white blood cells

At 24 hours after a single instillation, pulmonary exposure to either UPM or DEP led to a significant dose-dependent increase in BALF total white blood cell (WBC) counts (10 fold increase for $UPM_{200\mu g}$ vs vehicle, p for trend <0.0001, and 4-fold increase for $DEP_{200\mu g}$ vs vehicle, p for trend <0.0001), mainly driven by a neutrophil influx (Table 1). Total WBC and neutrophil counts in the UPM group, but not in the DEP group, were significantly higher than in the vehicle group, as early as 4h post-instillation, and peaked at 10h (Total WBC: 8-fold increase for $UPM_{100\mu g}$ *vs* vehicle; Neutrophils: increase from 0.9 (0.7-11.7) cells/µL for vehicle to 432 (342-476) cells/µL for $UPM_{100\mu g}$).

Table 1: Dose-response values for inflammatory parameters in broncho-alveolar lavage fluid, 24 hours after intratracheal administration of UPM or DEP in mice

	vehicle	UPM 25 µg	UPM 100 µg	UPM 200 µg	*correlation coefficient r*	*p for trend*
total WBC (/µL)	50 (40-70) *(18)*	120 (100-150) *(11)*	380 (320-420) *(11)*	510 (435-565) *(10)*	*0.952*	***<0.0001***
neutrophils (/µL)	0.4 (0.0-1.5) *(18)*	82 (70-86) *(11)*	331 (250-357) *(11)*	434 (356-487) *(10)*	*0.950*	***<0.0001***
macrophages (/µL)	50 (40-68) *(18)*	41 (29-53) *(11)*	64 (52-79) *(11)*	65 (58-77) *(10)*	*0.390*	***0.0051***
IL-6 (pg/mL)	0.6 (0.0-1.6) *(10)*	3.1 (1.6-6.3) *(5)*	12.0 (4.2-18.8) *(5)*	82.7 (61.3-92.9) *(5)*	*0.919*	***<0.0001***
KC (pg/mL)	0.6 (0.0-0.9) *(10)*	5.0 (3.7-9.1) *(5)*	7.3 (4.4-11.6) *(5)*	9.0 (7.2-18.8) *(5)*	*0.846*	***<0.0001***
MIP-1α (pg/mL)	0.0 (0.0-1.4) *(10)*	3.2 (1.5-7.7) *(5)*	10.6 (6.9-18.0) *(5)*	24.1 (21.5-46.3) *(5)*	*0.911*	***<0.0001***
MCP-1 (pg/mL)	5.3 (0.0-17.0) *(10)*	0.0 (0.0-7.2) *(5)*	0.0 (0.0-15.3) *(5)*	70.3 (44.9-89.3) *(5)*	*0.434*	***0.0302***
RANTES (pg/mL)	1.3 (0.0-3.4) *(10)*	1.2 (0.0-2.4) *(5)*	2.0 (0.0-2.9) *(5)*	9.1 (6.1-12.3) *(5)*	*0.503*	***0.0104***
		DEP 25 µg	**DEP 100 µg**	**DEP 200 µg**	***correlation coefficient r***	***p for trend***
total WBC (/µL)		75 (58-85) (6)	115 (103-155) (8)	220 (170-240) (9)	*0.863*	***<0.0001***
neutrophils (/µL)		28 (5-39) *(6)*	87 (57-113) *(8)*	156 (142-192) *(9)*	*0.920*	***<0.0001***
macrophages (/µL)		45 (43-49) *(6)*	39 (34-47) *(8)*	29 (25-49) *(9)*	*-0.383*	***0.012***
IL-6 (pg/mL)		3.5 (1.5-10.9) *(7)*	25.0 (8.8-53.8 *(7)*	80.9 (63.1-91.1) *(7)*	*0.898*	***<0.0001***
KC (pg/mL)		6.8 (1.1-26.9) *(7)*	13.7 (4.5-61.0) *(7)*	47.1 (17.2-73.0) *(7)*	*0.831*	***<0.0001***
MIP-1α (pg/mL)		5.2 (1.7-10.6) *(7)*	11.1 (8.0-17.4) *(7)*	30.1 (23.3-35.3) *(7)*	*0.932*	***<0.0001***
MCP-1 (pg/mL)		11.8 (8.8-13.4) *(7)*	13.4 (5.8-14.9) *(7)*	50.3 (26.0-53.3) *(7)*	*0.651*	***<0.0001***
RANTES (pg/mL)		3.5 (2.6-3.7) *(7)*	3.1 (2.6-4.0) *(7)*	3.4 (2.8-3.8) *(7)*	*0.360*	***0.0468***

Data are expressed as median (interquartile range) *(n)*. Significant p for trend values are highlighted in bold.

At all time points, cell numbers in the UPM group tended to be higher than for the DEP group (Fig. 1A and B). Overall, changes in macrophage numbers were modest at any dose and at any time-point after a single instillation (Table 1), despite obvious macrophage particle phagocytosis, as observed by light microscopy (Fig. 1F). When compared to a single instillation, repeated instillations further increased the levels of BALF macrophages after both UPM_{100nm} and DEP_{100nm} (both 2-fold increase for 3 instillations *vs* a single instillation, $p<0.01$) (Fig. 1C).

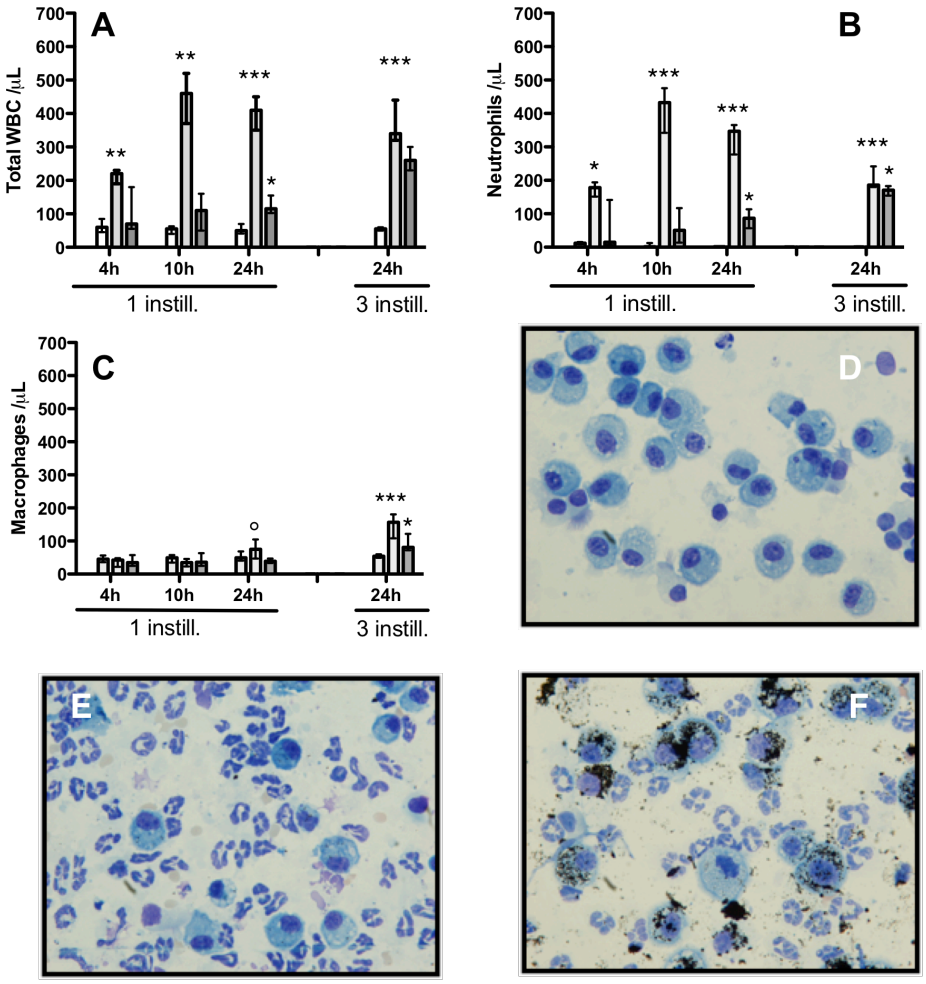

Figure 1: White blood cells (WBC) in broncho-alveolar lavage fluid.
WBC were measured in BALF at 4, 10 and 24 hours after a single intratracheal instillation (1 instill.), and at 24 hours after 3 repeated instillations (3 instill.) of vehicle (white bars, left), UPM_{100nm} (light grey bars, middle) or DEP_{100nm} (dark grey bars, right). A: total WBC count. B: neutrophil count. C: macrophage count. Values are represented as median+interquartile range ($n\geq5$ for each group). *:<0.05 UPM or DEP vs vehicle. **:<0.01 UPM or DEP vs vehicle. ***:<0.001 UPM or DEP vs vehicle. °:<0.05 UPM vs DEP.
Representative images of stained cytospin slides of WBC in BALF, 24 hours after single IT instillation of D: vehicle, E: UPM_{100nm}, F: DEP_{100nm}. Both UPM and DEP induce neutrophilic influx. Macrophagic ingestion of DEP is evident.

Pulmonary effects: cytokines

Mean IL-1β levels in BALF were around the detection limit (1.9 pg/mL) in all groups at any time point or any dose (data not shown).

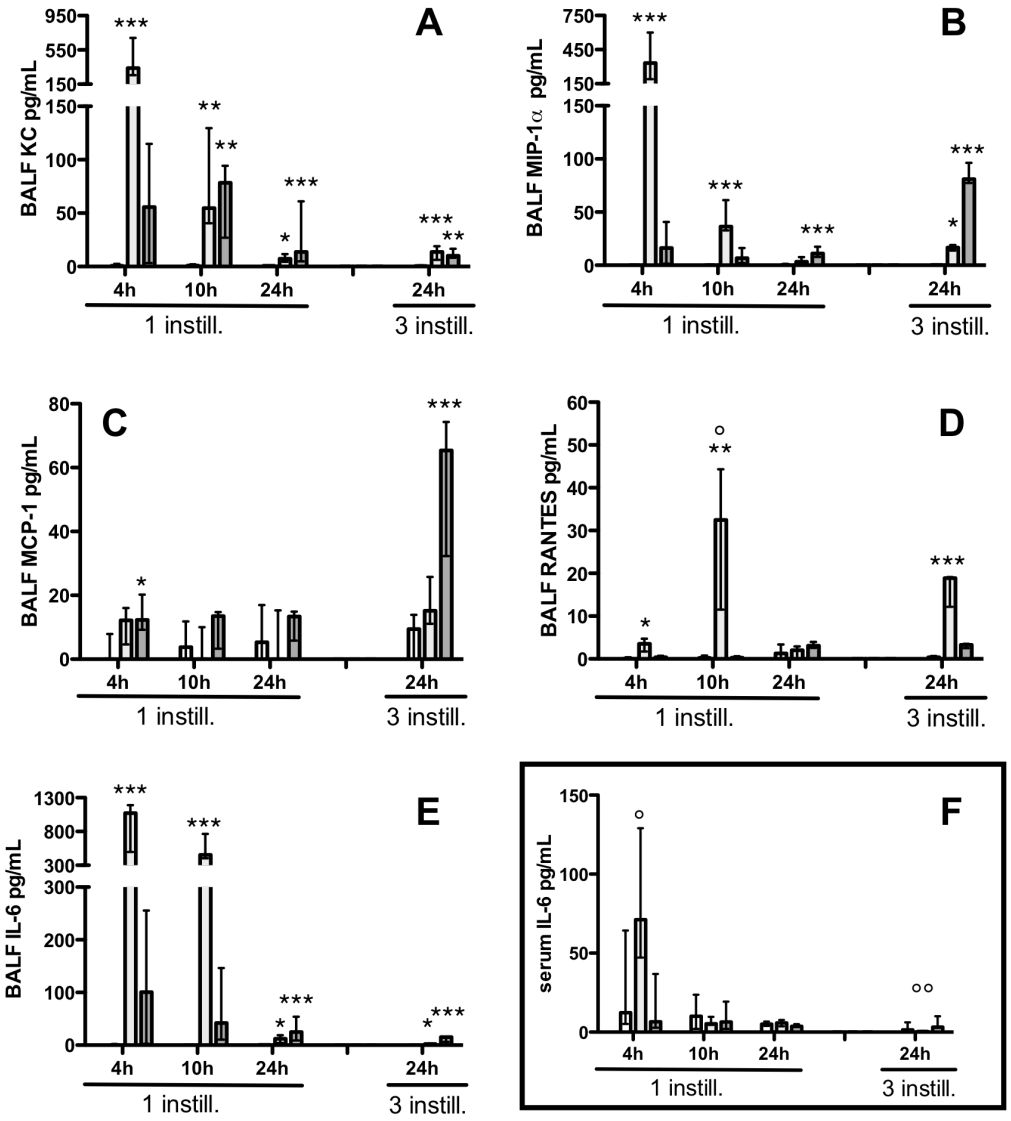

Figure 2: Cytokines in broncho-alveolar lavage fluid (BALF) and IL-6 in serum.
Cytokines were measured in BALF at 4, 10 and 24 hours after a single intratracheal instillation (1 instill.), and at 24 hours after 3 repeated instillations (3 instill.) of vehicle (white bars, left), UPM_{100nm} (light grey bars, middle) or DEP_{100nm} (dark grey bars, right). A: KC in BALF. B: MIP-1α in BALF. C: MCP-1 in BALF. D: RANTES in BALF. E: IL-6 in BALF. Serum levels of IL-6 are shown in F. Values are represented as median+interquartile range (n≥5 for each group). *:<0.05 UPM or DEP vs vehicle. **:<0.01 UPM or DEP vs vehicle. ***:<0.001 UPM or DEP vs vehicle. °:<0.05 UPM vs DEP. °°<0.01 UPM vs DEP.

Marked dose-dependent increases (p for trend<0.0001) were found in the levels of IL-6, KC, and MIP-1α for both UPM and DEP (Table 1).

Following a single instillation of $UPM_{100\mu g}$, levels of IL-6, KC and MIP-1α peaked at 4 hours, exceeding several hundred-fold the levels after instillation of vehicle, and several 10-fold those of $DEP_{100\mu g}$. These parameters returned to near-baseline levels at 24 hours (Fig. 2A,B,E). Upregulation of MCP-1 levels was only modest at any timepoint after a single instillation of 100 μg of either UPM or DEP (Fig. 2C), but more pronounced after a single instillation of 200 μg of either UPM or DEP (table 1) or after repeated instillation of $DEP_{100\mu g}$ (Fig. 2C). Concentrations of RANTES were only upregulated 10 hours after a single, or 24 hours after repeated instillations of $UPM_{100\mu g}$ (Fig. 2D).

Pulmonary effects: mRNA expression

A single exposure to $UPM_{100\mu g}$ induced an upregulation of both VCAM-1 and ICAM-1 at all time points, as compared to both vehicle and DEP, although only significantly at 4 hours for VCAM-1 and at 4 and 24 hours for ICAM-1. $DEP_{100\mu g}$ only upregulated ICAM-1 mRNA expression at 24 hours. This upregulation was no longer apparent after repeated instillations.

A single instillation of $UPM_{100\mu g}$, but not $DEP_{100\mu g}$, strongly upregulated tissue factor mRNA expression at 4 hours. No significant differences between groups were measured in the levels of thrombomodulin mRNA expression at any timepoint, but an upregulation was observed for all groups at 4 hours, apparently related to the IT instillation itself (Fig. 3).

Systemic effects: inflammation

At 4 hours, a trend towards a rapid and transient upregulation of serum IL-6 levels was induced by single exposure to $UPM_{100\mu g}$, that was not observed after $DEP_{100\mu g}$ (Fig. 2F). At 24 hours, neither $UPM_{100\mu g}$ nor $DEP_{100\mu g}$ upregulated IL-6 levels at any dose (data supplement Table E1). Repeated instillations did not enhance this effect (Fig. 2F).

No consistent changes were observed in the numbers of circulating WBC or blood platelets, nor in the serum concentrations of cytokines KC, MIP-1α, MCP-1 or RANTES at any dose (data supplement Table E1) or any time-point (on-line data supplement Fig. E1 and E2). Again, all values for serum IL-1β were around the detection limit (data not shown).

Systemic effects: thrombotic occlusion time

Pretreatment of non-instilled mice with LMWH lead to a considerable prolongation of venous, but not of arterial thrombotic occlusion time (Fig. 4A). This observation qualifies venous occlusion times as a valuable read-out, characteristic of venous thrombosis, i.e.

amongst others dependent on secondary haemostasis. In contrast, the absence of such prolongation in the arterial thrombosis model reflects the independence of secondary haemostasis parameters. Arterial thrombotic occlusion times therefore seem mainly determined by the vascular injury and activation of primary haemostasis.

At 24 hours after instillation, $UPM_{200\mu g}$ induced a significant shortening of the arterial thrombotic occlusion time, that was more pronounced than for $DEP_{200\mu g}$. In contrast, the venous thrombotic occlusion time was unaffected by both types of PM (Fig. 4B).

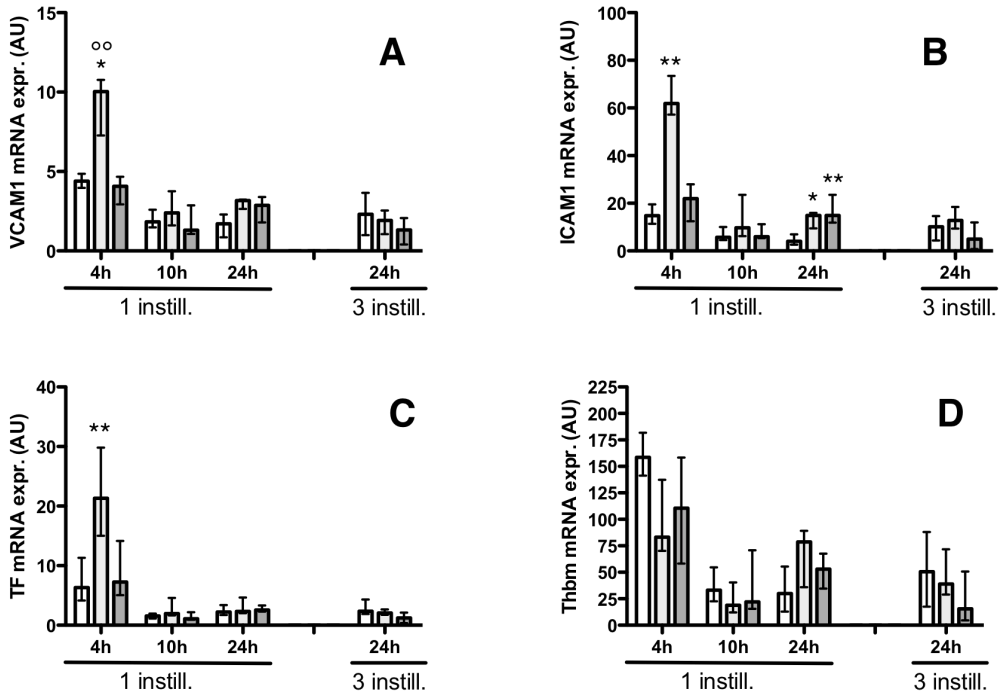

Figure 3: Pulmonary mRNA expression.
Pulmonary mRNA expression levels were measured at 4, 10 and 24 hours after a single intratracheal instillation (1 instill.), and at 24 hours after 3 repeated instillations (3 instill.) of vehicle (white bars, left), $UPM_{100\mu g}$ (light grey bars, middle) or $DEP_{100\mu g}$ (dark grey bars, right). A: VCAM-1. B: ICAM-1. C: tissue factor (TF). D: thrombomodulin (Thbm). C_t value of each parameter was expressed relatively to the C_t value of the house-keeping gene GaPDH for each separate sample, and results expressed as arbitrary units (AU). Values are represented as median+interquartile range ($n \geq 5$ for each group). *:<0.05 UPM or DEP vs vehicle. **:<0.01 UPM or DEP vs vehicle. °°<0.01 UPM vs DEP.

Systemic effects: plasma coagulation markers

Secondary hemostasis parameters aPTT, PT, fibrinogen, FVII and FVIII (1-stage coagulation and chromogenic assay) were measured at 24 hours after a single instillation (different doses) or after repeated instillations (100 μg doses) of PM (Table 2).

UPM, but not DEP, induced a significant dose-dependent prolongation of the aPTT ($r=0.5827$, p for trend<0.0001). A much milder prolongation was also observed after repeated

instillations of $UPM_{100\mu g}$, although not significant. No effect was seen on PT at any dose (Table 2). Plasma levels of fibrinogen, both a procoagulant and an acute phase protein, mildly increased with the dose, both for UPM and for DEP, and after repeated instillations of $DEP_{100\mu g}$, by about 20%.

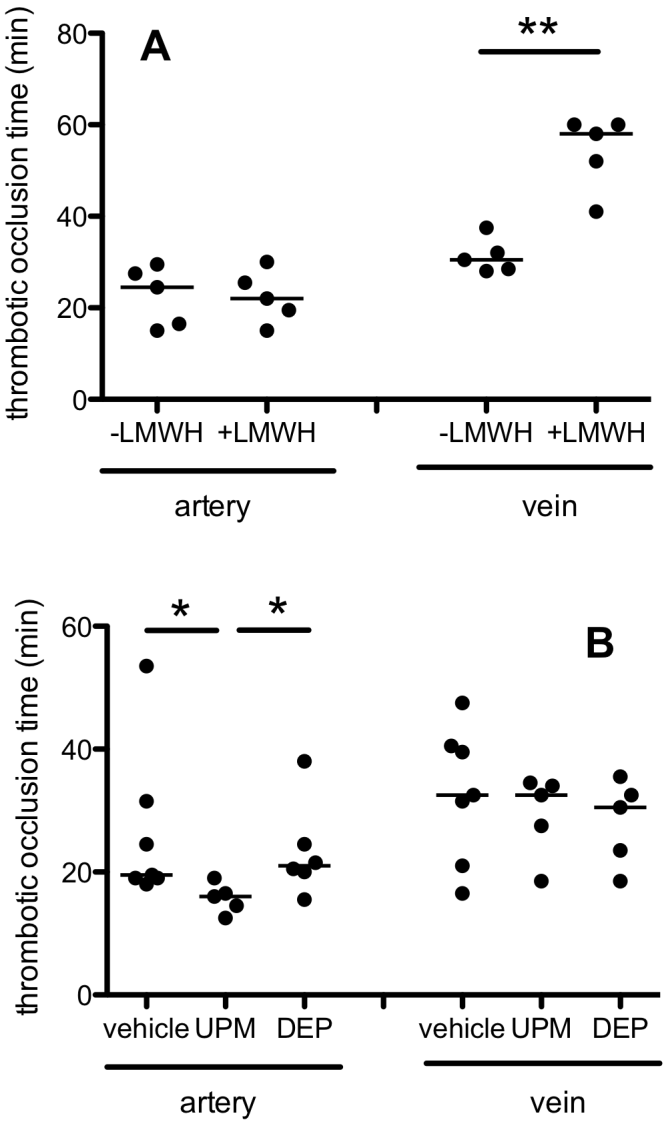

Figure 4: Arterial and venous thrombotic occlusion times.
Thrombotic occlusion times of arterial (artery) and venous (vein) thrombosis, upon $FeCl_3$-induced vascular injury of the mouse mesenteric circulation. A: Mice were treated with low molecular weight heparin (4 mg/kg) as indicated (controls: -LMWH; treated: +LMWH). B: Thrombotic occlusion times were measured in the mesenteric arteries and veins, 24 hours after a single intratracheal instillation of vehicle, $UPM_{200\mu g}$ or $DEP_{200\mu g}$ in mice. Values shown are the mean values of 2 separate measurements per mouse (n=5-7). Bars represent median values. *:<0.05 , **:<0.01

Similarly, FVII, measured via one-stage clotting assay increased by 20-25% after a single exposure to both UPM or DEP, with $r>0.4$ and $p<0.01$ (Table 2). This trend was not observed for the chromogenic assay and was not pronounced enough to shorten the PT. No consequent increase of FVIII levels was measured for exposure to UPM or DEP. Repeated instillations of UPM did not alter levels of FVII and FVIII.

Hepatic effects: mRNA expression

At 24 hours after a single dose or repeated doses of 100 μg of PM, we only observed a significant upregulation of the mRNA expression level of protein C, after a single instillation of $DEP_{100\mu g}$. Repeated exposure to either UPM or DEP did not alter mRNA expression (Table 3).

Discussion

To initiate mechanistic studies on the relationship between exposure to particulate matter and VTE, we have investigated inflammation, thrombogenicity and coagulation activation after exposing mice acutely and subacutely to two different types of relevant PM, administered intratracheally.

Both UPM and DEP dose-dependently increased WBC numbers (mainly neutrophils) and relevant cytokines in BALF. Overall, UPM induced a more potent pulmonary inflammatory response than DEP. This may be explained by the higher endotoxin content in the UPM sample (138.4 EU/mg of PM) than in the DEP sample (below detection limit of 0.05 EU/mg of PM). Previous experiments in our laboratory showed that the combined instillation of DEP and very low amounts of endotoxin, representative of the concentrations in UPM, resulted in enhanced cytokine expression in mice, with a semi-quantitative pattern highly similar to that observed for UPM (shown in Table E3 in the supplement).

UPM undergoes airborne condensation of lipopolysaccharides, metal ions and a mix of organic molecules on the surface, potentially all contributing to its adverse effects (35). We found previously that the UPM used in this study induced an upregulation of IL-6, IL-8 (equivalent to rodent KC) and MCP-1 in an *in-vitro* co-culture system mimicking the pulmonary cellular environment (36), and lead to elevated ICAM-1 and VCAM-1 on endothelial cells (37).

Table 2: Coagulation parameters in plasma after a single or repeated instillation of UPM or DEP in mice.

	1 instillation						3 instillations	
	vehicle	**UPM 25 µg**	**UPM 100 µg**	**UPM 200 µg**	***correlation coefficient r***	***p for trend***	**vehicle**	**UPM 100 µg**
aPTT (sec)	34.5 (33.5-36.6) *(18)*	37.3 (34.9-38.5) *(11)*	36.9 (35.2-41.6) *(11)*	40.3 (37.0-43.5) *(11)*	*0.5827*	***<0.0001***	35.2 (32.7-37.5) *(12)*	36.7 (35.9-38.2) *(7)*
PT (sec)	11.7 (11.0-12.0) *(16)*	11.1 (10.9-11.6) *(11)*	11.5 (11.1-11.7) *(11)*	11.4 (11.2-11.6) *(11)*	*-0.0590*	*0.6974*	11.1 (10.5-11.4) *(12)*	10.9 (10.7-11.6) *(7)*
$FVII_{coag}$ (%)	116 (106-130) *(13)*	104 (97-133) *(8)*	144 (129-154) *(8)*	139 (120-158) *(8)*	*0.4648*	***0.0038***	112 (104-134) *(11)*	134 (125-154) *(7)*
$FVII_{chrom}$ (%)	155 (134-174) *(18)*	145 (142-165) *(11)*	159 (146-174) *(10)*	173 (143-204) *(11)*	*0.2134*	*0.1367*	ND	ND
$FVIII_{coag}$ (%)	124 (114-132) *(18)*	126 (114-136) *(11)*	127 (122-138) *(11)*	117 (114-138) *(11)*	*0.09612*	*0.5022*	122 (108-129) *(11)*	133 (124-145) *(7)*
$FVIII_{chrom}$ (%)	118 (106-134) *(16)*	154 (112-181) *(11)*	151 (121-173) *(11)*	160 (125-205) *(10)*	*0.3059*	***0.0345***	ND	ND
Fbg (g/L)	6.85 (6.00-7.70) *(15)*	7.78 (6.20-8.65) *(11)*	7.93 (7.18-9.22) *(11)*	8.14 (6.44-9.41) *(10)*	*0.3358*	***0.021***	7.02 (5.83-7.50) *(11)*	6.44 (6.12-6.79)° *(7)*
		DEP 25 µg	**DEP 100 µg**	**DEP 200 µg**	***correlation coefficient r***	***p for trend***		**DEP 100 µg**
aPTT (sec)		34.9 (31.5-36.3) *(7)*	34.0 (32.4-35.9) *(8)*	33.8 (31.9-35.8) *(9)*	*-0.1409*	*0.3735*		36.1 (33.6-40.0) *(5)*
PT (sec)		11.5 (10.4-12.6) *(7)*	11.3 (11.0-11.8) *(8)*	11.4 (10.1-12.4) *(9)*	*-0.1354*	*0.4048*		10.6 (10.2-10.9)
$FVII_{coag}$ (%)		138 (118-174) *(7)*	117 (107-157) *(8)*	141 (129-168) *(9)*	*0.428*	***0.0082***		106 (103-128) *(5)*
$FVII_{chrom}$ (%)		163 (155-165) *(7)*	154 (140-158) *(8)*	165 (156-174) *(8)*	*0.114*	*0.4779*		ND
$FVIII_{coag}$ (%)		138 (124-147) *(7)*	125 (113-135) *(8)*	132 (126-147) *(9)*	*0.3176*	***0.0404***		126 (114-159) *(5)*
$FVIII_{chrom}$ (%)		126 (91-157) *(6)*	124 (117-144) *(7)*	124 (115-162) *(9)*	*0.1576*	*0.3448*		ND
Fbg (g/L)		8.06 (7.12-9.32) *(7)*	6.62 (6.02-7.39) *(7)*	8.21 (7.49-8.80) *(9)*	*0.3935*	***0.0145***		**8.18 (7.95-9.58)**** *(5)*

Data are expressed as median (interquartile range) *(n)*. **:<0.01 DEP vs vehicle. °:<0.05 UPM vs DEP. Values significantly different from the vehicle-treated group and significant p for trend values are highlighted in bold. ND: not determined.

Table 3: Hepatic mRNA expression after a single or repeated instillation of UPM or DEP in mice.

	1 instillation			3 instillations		
	vehicle	**UPM 100 µg**	**DEP 100 µg**	**vehicle**	**UPM 100 µg**	**DEP 100 µg**
FVII mRNA (AU)	2.25 (1.73-3.03) *(11)*	3.05 (2.46-3.41) *(5)*	2.29 (2.09-2.63) *(6)*	2.36 (2.11-2.68) *(11)*	2.15 (1.79-2.31) *(6)*	2.12 (1.88-2.50) *(5)*
Fbg β mRNA (AU)	71.46 (40.5-124.2) *(11)*	72.11 (71.07-98.06)° *(5)*	39.82 (32.61-46.76) *(6)*	68.61 (41.53-83.76) *(11)*	80.32 (73.62-93.11) *(6)*	38.34 (28.15-125.60) *(5)*
AT mRNA (AU)	327.4 (279.9-345.4) *(11)*	304.9 (291.8-327.3) *(5)*	343.3 (316.9-498.4) *(6)*	307.8 (252.8-393.7) *(11)*	233.0 (208.6-269.2) *(6)*	355.7 (245.1-399.9) *(5)*
ProtC mRNA (AU)	23.73 (21.34-25.16) *(11)*	24.53 (22.36-26.51) *(5)*	**27.19 (25.67-31.87)**** *(6)*	26.93 (20.49-29.37) *(11)*	17.66 (16.43-23.48) *(6)*	26.77 (23.37-29.73) *(5)*

Data are expressed as median (interquartile range) *(n)*.
For mFor mRNA expression levels, Ct value of each parameter was expressed relatively to the Ct value of the house-keeping gene GaPDH for each separate sample, and expressed as arbitrary units (AU)). Fbg β: fibrinogen β-chain. AT: antithrombin. ProtC: protein C.
**:<0.01 DEP vs vehicle. °:<0.05 UPM vs DEP. Values significantly different from the vehicle-treated group are highlighted in bold.

Despite obvious pulmonary inflammation for both common types of particles, we found no substantial systemic inflammation. Published results of systemic inflammation after acute or chronic exposure to PM in man are conflicting. Ambient PM has been associated with higher levels of WBC (38), IL-6 (18) and hs-CRP (16) both in healthy humans and in at-risk populations, but systemic inflammation was not evident in all epidemiological studies (24). Likewise, although some controlled exposure studies showed associations between exposure to fairly high concentrations of PM and increased levels of circulating neutrophils (39), platelet-WBC aggregates (21), IL-6 and TNFα (40), others failed to observe increases in circulating WBC (15, 41), IL-6 (41) or CRP (22, 23, 41), within 24 hours after exposure.

Exposure to UPM and DEP induced mild changes in both procoagulant (tissue factor mRNA, FVII protein, FVIII protein and fibrinogen protein), as well as in anticoagulant (protein C mRNA) pathways. The rise of FVII in the UPM and DEP groups was observed when analyzed via one-stage clotting assay, but not with a chromogenic assay. This is suggestive of exposure-related FVII activation, rather than de novo FVII synthesis and release in the circulation. Despite increased levels of the acute phase proteins FVIII and fibrinogen in the UPM-treated group, a contradictory dose-dependent prolongation of the aPTT clotting time was observed. One possible explanation for this apparent discrepancy may be the recently demonstrated prolongation of the aPTT in human plasma upon addition of CRP, as a result of its binding to negatively charged phospholipids (42). In mice, CRP is not a strong acute phase protein, but more relevant acute phase proteins, such as serum amyloid a (SAA), also bind phospholipids (43) and may cause similar interference.
In general, procoagulant changes measured were mild and were similar between UPM and DEP, even at the highest doses (200 μg). At the lower dose of 25 μg, which is more representative for daily inhaled doses in humans (i.e. comparative dose of ±35 μg/mouse) (29) no relevant procoagulant changes were observed.
The mild activation of secondary haemostasis is too weak to influence experimental venous thrombosis in mice, in contrast to the more prothrombotic effect of UPM on arterial thrombosis. Because arterial thrombogenicity in the model used is determined primarily by the intensity of the vascular lesion, platelet recruitment and aggregation (34), these findings are compatible with UPM-induced platelet activation. Blood

platelet activation was not directly measured in this study, but previously, our group has provided ample evidence of blood platelet activation upon short-term PM-exposure in hamsters (6, 44), mice (7) and recently in humans (38). Thus, while short-term increases in PM exposure affect pathways promoting arterial thrombosis, to trigger a risk of venous thrombosis a more chronic exposure to elevated levels of PM seems required, via pathways only understood in part, at the present time. This is corroborated by epidemiological findings in which the risk for DVT was only associated with the mean PM concentration over a one year period, and not with any shorter time-point (10). Likewise, shortenings in PT were only observed for increases in mean PM concentration of 1 month or more (13, 14).

Surprisingly few experimental animal studies have hitherto been published on the association between air pollution and secondary haemostasis activation. Mutlu et al. reported a pronounced prothrombotic phenotype in wild type, but not in IL-6 knock-out mice, characterized by shortenings in bleeding time, PT and aPTT, and relatively high increases in the levels of circulating blood platelets, FVII, FVIII, FX and fibrinogen, 24 hours after IT instillation of 10 μg of UPM, a dose 2.5 to 20 times smaller than in the present study (29). Surprisingly, the pulmonary WBC influx in their study almost completely consisted of macrophages, in contrast to the predominant neutrophil influx in our study and many others (7, 45-48). This remarkable difference in pulmonary WBC influx, possibly due to the type of particles used, may explain why the study by Mutlu et al. stands out among other studies on the procoagulant effects of PM in mice. Indeed, a few other experimental studies in rodents observed procoagulant changes in levels of fibrinogen, activated protein C, tissue factor pathway inhibitor (TFPI) or PAI-1, but only at doses of 100 μg or higher of PM per mouse (26, 27). In addition, exposure of rats to concentrated PM from New York City air did not alter levels of fibrinogen, FVII, PAI-1 or thrombin-antithrombin complexes (TAT) (28).

Despite the high similarity of haemostasis between humans and mice, differences do exist (49). We therefore can not exclude that the modest effects observed on secondary haemostasis in the present work are linked to the use of a mouse model, which, indeed, seems less responsive to DEP than hamsters (6, 44).

In conclusion, UPM induced more pronounced pulmonary inflammation than DEP in healthy mice, likely attributable to a higher endotoxin content, but triggered weak systemic inflammation and mild secondary haemostatic activation only, even at high exposure. We therefore doubt that acute exposure studies in healthy mice will clarify the association in man between PM exposure and the risk of venous thrombosis. Chronic exposure studies in healthy and cardiovascular compromised mice, in conjunction with observational studies in cohorts of risk patients, are more likely to provide understanding of the relationship between long-term exposure to pollutant PM, systemic inflammation and secondary haemostasis.

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Conflict of interest:

The authors report no conflict of interest.

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Data supplement

Materials and methods:

The composition of the UPM used in the main article is given in Table S1.

Measurement of circulating blood cells (Table S2 and Fig. S1) and quantitative cytokine analysis using multiplex bead assay (Table S2 and Fig. S2) after single or repeated instillations of UPM or DEP were performed as specified in the main article.

In preliminary experiments, we aimed to investigate the contributing role of endotoxin (lipopolysaccharide, LPS) to PM-induced lung inflammation (Table E3). To this end, 8- tot 10-week old male C57Bl6/n mice were exposed via a single IT instillation to LPS alone (3 ng/mouse, Sigma-Aldrich, Bornem, Belgium), DEP alone (25 μg/mouse, National Institute for Standards and Technology, Gaithersburg, USA, SRM 1650b), a combination of LPS and DEP (3 ng/mouse and 25 μg/mouse, respectively) or UPM alone (25 μg/mouse, Mexico City, Mexico, as specified in the main article) resuspended in vehicle (NaCl 0.9%, containing 0.1% Tween 80). Controls consisted of vehicle-treated mice and sham mice. The dose of LPS (3 ng/mouse) was chosen to be comparative to the amount of LPS present in 25 μg/mouse of UPM. Based on the manufacturer's information (endotoxin content of LPS: 500,000 EU/mg), a dose of LPS of 3 ng/mouse corresponds to 1.5 EU/mouse. Based on the endotoxin content of the UPM (138.4 EU/mg), a dose of UPM of 25 μg/mouse corresponds to 3.5 EU/mouse. DEP used in this preliminary experiment were different from the DEP used in the main article, and results are, therefore, not strictly comparable. However, as for the DEP used in the main article, LPS content of these DEP also was below the detection limit of 0.05 EU/mg of PM.

At 24 hours post-instillation, BALF was obtained as specified in the main article. BALF of 5 mice per experimental group was pooled for semi-quantitative analysis of the cytokine profile using the Proteome Profiler, Mouse Cytokine Array kit (R&D systems Inc, Minneapolis, USA). The obtained autoradiographs were scanned and analyzed using the ImageJ program (National Institute of Health, Bethesda, USA). The relative density of each dot was calculated in relation to the positive internal controls of the membranes and semi-quantitatively subcategorized.

Results:

Table S1: Elemental composition and lipopolysaccharide (LPS) content of UPM (1)									
Elements		**Si**	**Ca**	**Al**	**Fe**	**K**	**S**	**Ti**	**Cl**
	µg/mg of PM	69	25	23	12	4	4	1.8	1
		P	**Zn**	**Mn**	**Cr**	**Cu**	**Pb**	**V**	**Ni**
	µg/mg of PM	1	1	0.4	0.1	0.1	0.1	0.1	<0.1
LPS	EU/mg*	138.4							
* EU: endotoxin units									

Table S2: Dose-response values for blood cells and serum cytokines

	vehicle	UPM 25 µg	UPM 100 µg	UPM 200 µg	correl. coeff. r	p for trend
total WBC (10^3/µL)	4.75 (3.73-5.60) *(17)*	3.34 (2.74-3.73) *(11)*	3.04 (2.32-3.92) *(11)*	3.94 (3.57-5.40) *(11)*	-0.150	0.2978
neutrophils (10^3/µL)	1.05 (0.83-1.61) *(17)*	0.89 (0.63-1.05) *(11)*	0.54 (0.48-0.90) *(11)*	0.76 (0.59-0.91) *(11)*	-0.393	**0.0047**
monocytes (10^3/µL)	0.04 (0.02-0.11) *(17)*	0.09 (0.04-0.14) *(11)*	0.05 (0.03-0.07) *(11)*	0.05 (0.03-0.06) *(11)*	-0.005	0.9715
lymphocytes (10^3/µL)	3.27 (2.31-4.54) *(17)*	2.08 (1.40-2.67) *(11)*	2.37 (1.47-2.55) *(11)*	3.11 (2.65-3.65) *(11)*	-0.0989	0.4945
BP (10^3/µL)	1205 (1113-1280) *(17)*	1225 (1135-1305) *(11)*	1130 (1095-1360) *(11)*	1305 (1225-1455) *(11)*	0.2110	0.1414
IL-6 (pg/mL)	5.0 (4.0-6.7) *(9)*	7.7 (4.2-10.5) *(5)*	5.9 (3.7-7.7) *(5)*	5.7 (3.7-6.6) *(5)*	0.0353	0.8698
KC (pg/mL)	6.1 (5.6-7.4) *(9)*	12.1 (4.0-21.6) *(5)*	11.2 (6.1-11.9) *(5)*	8.5 (7.3-10.7) *(5)*	0.328	0.8698
MIP-1α (pg/mL)	3.0 (1.8-4.0) *(9)*	2.8 (2.4-15.5) *(5)*	2.4 (1.8-3.9) *(5)*	2.6 (2.2-3.0) *(5)*	-0.173	0.4186
MCP-1 (pg/mL)	25.6 (23.4-28.7) *(9)*	25.2 (22.4-60.2) *(5)*	25.0 (14.5-32.1) *(5)*	28.3 (10.4-34.2) *(5)*	-0.049	0.8221
RANTES (pg/mL)	64.8 (48.7-117.3) *(9)*	141.8 (122.1-154.7) *(5)*	166.8 (75.2-178.8) *(5)*	104.3 (87.0-156.9) *(5)*	0.391	0.0592
		UPM 25 µg	**UPM 100 µg**	**UPM 200 µg**	**correl. coeff. r**	**p for trend**
total WBC (10^3/µL)		3.30 (1.27-4.67) *(7)*	3.27 (3.06-4.09) *(8)*	4.14 (2.55-4.81) *(9)*	-0.300	0.0541
neutrophils (10^3/µL)		1.91 (1.60-2.30) *(7)*	1.08 (0.96-1.65) *(8)*	1.57 (1.22-1.77) *(9)*	0.261	0.0996
monocytes (10^3/µL)		0.11 (0.03-0.15) *(7)*	0.05 (0.02-0.07) *(8)*	0.02 (0.01-0.07) *(9)*	-0.159	0.3260
lymphocytes (10^3/µL)		2.92 (0.61-4.39) *(7)*	3.06 (2.90-3.79) *(8)*	3.64 (2.34-4.55) *(9)*	-0.0112	0.9448
BP (10^3/µL)		1355 (1220-1385) *(7)*	1161 (1125-1350) *(8)*	1315 (1270-1405) *(9)*	0.0758	0.6378
IL-6 (pg/mL)		4.3 (3.7-16.1) *(7)*	3.7 (3.2-5.1) *(6)*	6.3 (4.7-6.7) *(7)*	-0.0038	0.9843
KC (pg/mL)		7.4 (5.7-9.9) *(7)*	6.3 (5.0-9.8) *(6)*	9.9 (6.3-10.5) *(7)*	0.243	0.2035
MIP-1α (pg/mL)		3.1 (2.6-4.2) *(7)*	3.4 (2.5-5.0) *(6)*	3.3 (2.7-4.1) *(7)*	0.151	0.4357
MCP-1 (pg/mL)		27.8 (20.1-42.5) *(7)*	29.9 (25.8-33.7) *(6)*	27.8 (24.7-43.9) *(7)*	0.210	0.274
RANTES (pg/mL)		58.6 (46.1-70.6) *(7)*	66.8 (52.3-73.5) *(6)*	61.9 (47.9-67.8) *(7)*	-0.100	0.6060

Data are expressed as median (interquartile range) *(n)*.
Significant p for trend values are highlighted in bold.

Table S2 shows that UPM and DEP hardly induce any changes in blood inflammatory cells and circulating cytokines, despite important changes in BALF (see main article). The only remarkable finding is the drop in circulating neutrophils, which possibly migrate to the lung, upon UPM exposure.

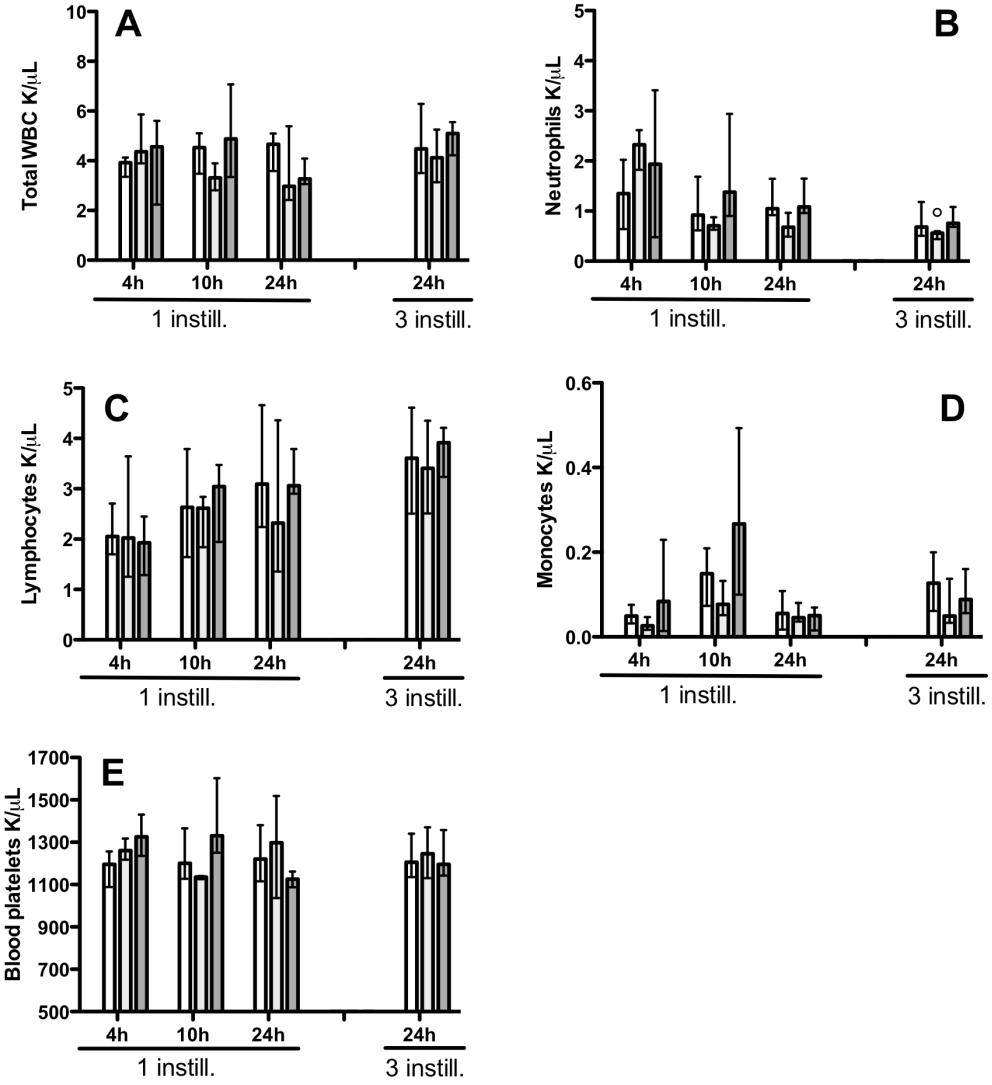

Figure S1: Circulating WBC and blood platelet numbers.
WBC and blood platelets were measured in blood at 4, 10 and 24 hours after a single instillation (1 instill.), and at 24 hours after 3 repeated instillations (3 instill.) of vehicle (white bars, left), UPM_{100mg} (light grey bars, middle) or DEP_{100mg} (dark grey bars, right). A: total WBC count. B: neutrophil count. C: lymphocyte count. D: monocyte count. E: blood platelet count. Values are represented as median+interquartile range ($n \geq 5$ for each group). °:<0.05 UPM vs DEP.
No relevant significant changes in cell numbers occurred.

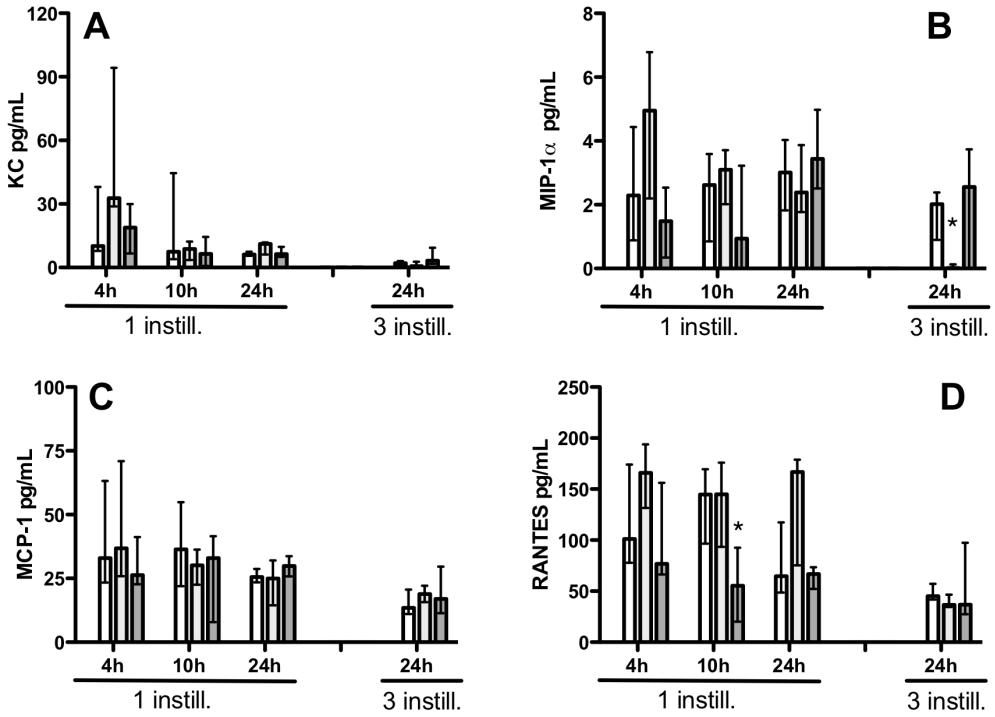

Figure S2: Inflammatory cytokines in serum.
KC, MIP-1a, MCP-1 and RANTES were measured in blood at 4, 10 and 24 hours after a single instillation (1 instill.), and at 24 hours after 3 repeated instillations (3 instill.) of vehicle (white bars, left), $UPM_{100_{rg}}$ (light grey bars, middle) or $DEP_{100_{rg}}$ (dark grey bars, right). A: KC. B: MIP-1a. C: MCP-1. D: RANTES. Values are represented as median+interquartile range ($n \geq 5$ for each group). *:<0.05 UPM or DEP vs vehicle. No relevant significant changes occurred in cytokine levels.

	Sham	NaCl	DEP	LPS	LPS +DEP	UPM
BLC						
C5a						
G-CSF						
GM-CSF						
I-309						
Eotaxin						
sICAM-1						
IFN-y						
IL-1a						
IL-1b						
IL-1ra						
IL-2						
IL-3						
IL-4						
IL-5						
IL-6						
IL-7						
IL-10						
IL-13						
IL-12 p70						
IL-16						
IL-17						
IL-23						
IL-27						
IP-10						
I-TAC						
KC						
M-CSF						
JE						
MCP-5						
MIG						
MIP-1a						
MIP-1b						
MIP-2						
RANTES						
SDF-1						
TARC						
TIMP-1						
TNF-a						
TREM-1						

relative concentration:

>80
40-80
20-40
5-20
<5

Table S3: Cytokine profile in broncho-alveolar lavage fluid
Cytokine concentrations in sham mice and in mice exposed to vehicle, UPM alone, DEP alone, LPS alone or the combination of DEP and LPS (n=5 per group) are expressed as a semi-quantitative fold-increase, relative to an internal positive control on each analytical membrane. The cytokine profile in mice exposed to the combination of DEP and LPS is highly similar to the profile in mice exposed to UPM alone.

Supplemental reference

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CHAPTER 2

Air Pollution-associated procoagulant Changes: Role of circulating Microvesicles

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Abstract

Background: Epidemiological studies suggest an association between exposure to particulate matter (PM) in air pollution and risk of venous thromboembolism (VTE).

Objectives: To investigate the underlying pathophysiological pathways linking PM exposure and VTE.

Methods: We assessed potential associations between PM exposure and coagulation and inflammation parameters, including circulating microvesicles, in a group of 233 patients with diabetes.

Results: The numbers of circulating blood platelet-derived and annexin V-binding microvesicles were inversely associated with the *current* levels of $PM_{2.5}$ or PM_{10}, measured on the day of sampling. Recent past exposure to PM_{10}, up to 1 week prior to blood sampling, estimated at the patients' residential addresses, was associated with elevated high-sensitivity C-reactive protein (CRP), leukocytes and fibrinogen, as well as with tissue factor-dependent procoagulant changes in thrombin generation assays. When longer windows of past exposure were considered, up to 1 year preceding blood sampling, procoagulant changes were evident from the strongly increased numbers of red blood cell-derived circulating microvesicles and annexin V-binding microvesicles, but they no longer associated with tissue factor. Past PM exposure was never associated with activated partial thromboplastin time (aPTT), prothrombin time (PT), or factor (F) VII, FVIII, FXII or D-dimers. Residential distance to a major road was only marginally correlated with procoagulant changes in FVIII and thrombin generation.

Conclusions: Increases in the number of microvesicles and in their procoagulant properties, rather than increases in coagulation factors per se, seem to contribute to the risk of VTE, developing during prolonged exposure to air pollutants.

Introduction

Ambient environmental air pollutants include gaseous and particulate components. Considering a large body of evidence, the American Heart Association scientific statement on 'Air Pollution and Cardiovascular Disease' concluded that both short-term and long-term exposure to the particulate component (particulate matter, PM) are associated with increased mortality and cardiovascular disease (1). In addition to the well-recognized air pollution-related adverse effects on the arterial vascular system (2-8), recent epidemiological evidence also suggests an association between PM exposure and venous thromboembolism (VTE). Thus, a higher risk for deep vein thrombosis (DVT) was associated with increased annual mean levels of PM with a mean aerodynamic diameter smaller than 10 μm (PM_{10}) in the residential area of the study subjects (9). In the same study population, living near major traffic roads was also associated with an increased risk of DVT, even after controlling for the community-level PM pollution (10). These initial epidemiological findings by the group of Baccarelli were recently confirmed in a time-series analysis in Chile, demonstrating an association between PM exposure and hospital admission for VTE (11), although also challenged by others (12, 13).

The pathophysiological mechanisms explaining the observed link between PM exposure and VTE remain largely unknown. Although increases in the levels of coagulation factors seem the most likely explanation, published data for this interpretation are conflicting and unconvincing. In fact, disappointingly few studies reported on positive associations between air pollution exposure and increased levels of coagulation factors, and estimated effect sizes for the reported associations are relatively small (14-23). Therefore, the observed increases in coagulation factors are unlikely to be (solely) responsible for an increased venous thrombogenicity.

A potential role for microvesicles (also called microparticles, a term we prefer to avoid in the context of pollution by particles) has been suggested (14, 24, 25). Microvesicles are circulating vesicles with a mean diameter smaller than 1 μm that are released from stimulated or apoptotic cells in the vascular bed. Negatively charged phospholipids and tissue factor (TF) on their membranes create a procoagulant surface on which coagulation factors can bind and be activated to promote

coagulation (26). Elevated numbers of circulating microvesicles have been demonstrated in patients with VTE (27, 28). A direct link between air pollution and an elevation in the concentration of circulating microvesicles or their procoagulant potential has hitherto never been shown in humans.

In the present study, we hypothesized that microvesicles, through their procoagulant potential, could represent a missing link between air pollution exposure and VTE. We, therefore, investigated associations between PM exposure and markers of inflammation and coagulation, with a focus on microvesicles and microvesicle-dependent coagulation assays. We investigated these associations in an a priori susceptible population of patients with diabetes, because diabetic subjects are more sensitive to the deleterious effects of PM during air pollution (29).

Materials and methods

Study population

Persons with either type 1 or type 2 diabetes were recruited from the diabetes outpatient clinic at the University Hospital Leuven, Belgium, as a new cohort of patients, different from the cohort of our previous studies (30, 31). These patients visit the diabetes clinic as part of their routine follow-up. They were included if they were 18 years or older, current (for >6 months) non-smokers and not on anticoagulant therapy. Inclusion was done on different days from February 2010 through April 2010. Of 402 patients contacted, 339 agreed to participate (84% participation rate). We excluded 106 patients because of current smoking (n=74), anticoagulant therapy (n=16), accidental lack of blood samples (n=10) or other reasons (n=6). Thus, the final study population consisted of 233 included patients (Fig. 1). On the study day, patients completed a questionnaire through a personal interview to collect information on age, occupation, socioeconomic status, exposure to environmental tobacco smoke, alcohol use, use of medication, use of oral contraception, menopausal status, place of residence and means of transportation to the hospital. Socioeconomic status was encoded and condensed into a scale with scores ranging from 1 to 3, based on educational level. The Ethics Review Board of the Medical Faculty of the University of Leuven (KULeuven) approved the study. Participants gave informed consent at recruitment.

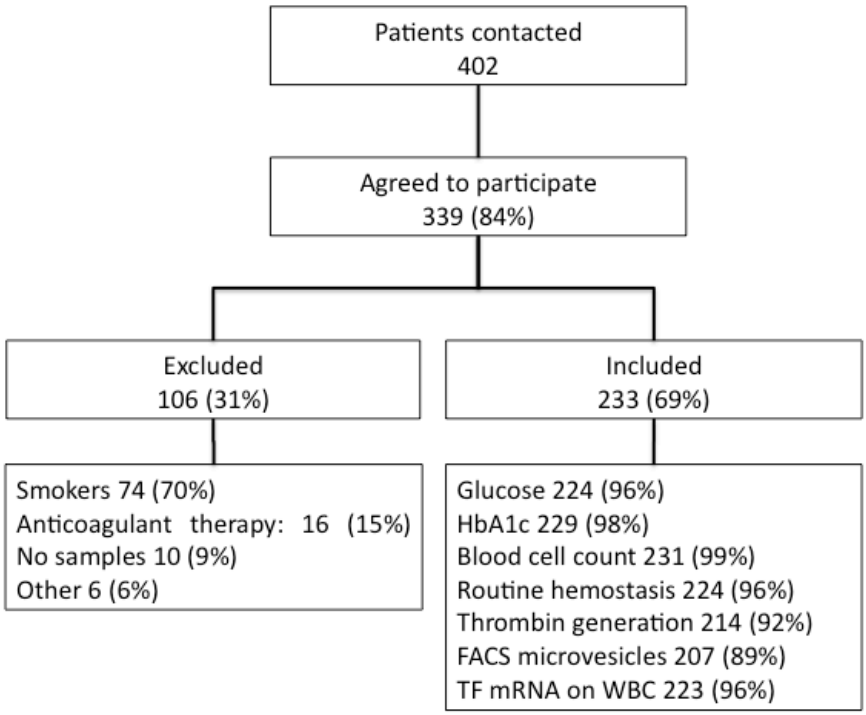

Figure 1. Flowchart of the study population
The study population was consecutively recruited from the diabetes outpatient clinic at the University Hospital Leuven. The lower right box shows the percentage of samples that were measured per group of analyses. HbA1c=glycated hemoglobin, FACS= flow cytometric analysis, WBC=white blood cells in blood.

Exposure assessment

Current exposure

A portable laser-operated aerosol mass analyzer (Aerocet 531, Met One Instruments Inc, Grant Pass, OR, USA), previously calibrated against a local monitoring station (Flemish Environmental Agency, Borgerhout, Belgium) (31), was used to measure *current* $PM_{2.5}$ and PM_{10} concentrations in the hospital waiting room, one to two hours before the patient's participation in the study. In general, patients stayed in the waiting room and the neighboring examination room for at least one hour.

Subacute, subchronic and chronic exposure

The regional background level of PM_{10} at each patient's residential address was calculated using a kriging interpolation method. This model provides interpolated

PM_{10} values from the Belgian telemetric air quality network in 4x4 km grids (see Fig. 2). The interpolation is based on a detrended kriging interpolation model that uses land cover data obtained from satellite images (Corine land cover data set, European Environment Agency, 2000) (32). Regional background levels of $PM_{2.5}$ are not available in Belgium. Mean residential PM_{10} values were measured for different time windows, and classified in 3 categories of exposure: 1) *subacute*: mean residential PM_{10} values on the day of blood sampling ('day 0'), on the first ('day -1'), the second ('day -2') or the third ('day-3') day before blood sampling; 2) *subchronic*: mean residential PM_{10} values over the preceding week ('mean 1 week') or month ('mean 1 month'); 3) *chronic*: mean residential PM_{10} values over the preceding 3 months ('mean 3 months'), 6 months ('mean 6 months') or 12 months ('mean 1 year'). Distances from the home address to major roads (N-road, a major traffic road or E-road, a motorway/highway) also reflect chronic PM exposure and were calculated through geocoding (the shortest distance being set at 10 meters).

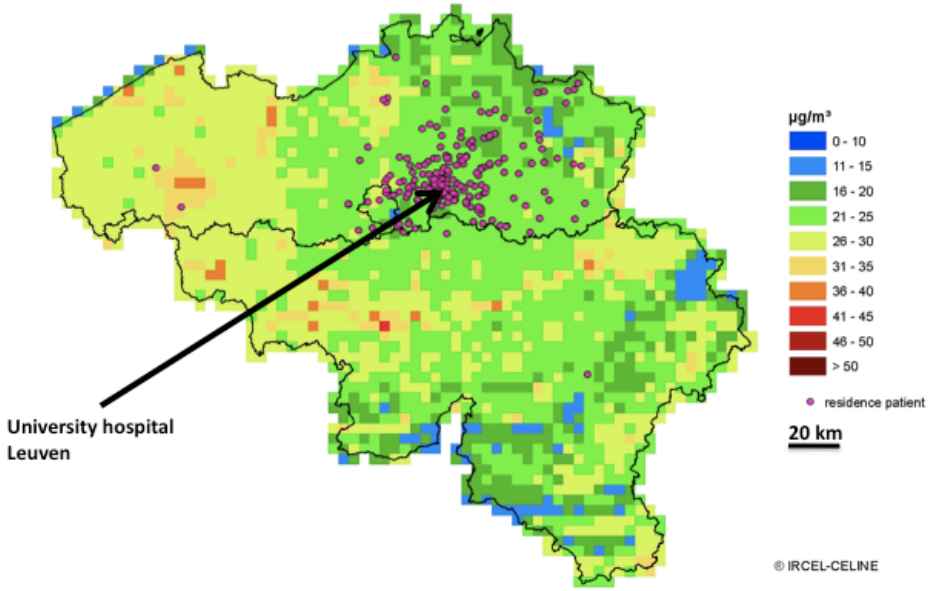

Figure 2. Location of residences of study subjects
Map of Belgium reporting location of the study center (University hospital, Leuven, Belgium) and location of the residences of the study subjects. Background colors represent mean annual PM_{10} concentrations for 2010 in 4x4 km grids

Clinical measurements

All laboratory tests were performed without knowledge of the subject's exposure data. A detailed description and validation of the assays used in this study to identify and characterize microvesicles is given in the online data supplement. Figure S1 schematically summarizes the physiological role of microvesicles in the coagulation cascade and illustrates which assays were performed in this study to evaluate microvesicles. In brief, microvesicle quantification was performed by flow cytometry (see below). To evaluate their procoagulant potential, surface expression of negatively charged phospholipids was evaluated by flow cytometry and surface expression of functional tissue factor by thrombin generation assays (TGA, see below).

Blood

All blood samples were collected in a restricted time window in the diabetes outpatient clinic (median hour of sampling: 01.50 p.m.), thus reducing the possibility of confounding by circadian rhythms of some parameters. Non-fasting blood samples were collected using a 21 gauge needle (Terumo, Leuven, Belgium) on EDTA, on sodium fluoride/oxalate, or on sodium citrate (3.8%) tubes (all BD Vacutainer, BD Biosciences, Erembodegem, Belgium). Analysis of blood cell counts and glucose and glycated hemoglobin (HbA1c) levels were performed on fresh full blood or plasma samples. For all other parameters, plasma was stored immediately at -80°C for future batch analysis. Citrated samples were centrifuged according to two different protocols: for biochemical analyses, tubes were centrifuged once at 3000 x g. For the analysis of microvesicles, both by flow cytometry and by TGA, tubes were first centrifuged for 10 minutes at 1900 x g, followed by a second centrifugation step of 20 minutes at 1900 x g to obtain blood platelet-depleted but microvesicle-rich plasma. All plasma samples were centrifuged within one hour after collection.

Blood cell counts and routine biochemical analysis

Blood cell counts, coagulation parameters, glucose levels, glycated hemoglobin (HbA1c) and high-sensitivity CRP (hsCRP) were measured according to standard clinical procedures on automated analyzers. The following "traditional" coagulation parameters were measured: activated partial thromboplastin time (aPTT), prothrombin time (PT), factor (F) VII, FVIII, FXII, fibrinogen and D-dimers.

Thrombin generation assays (TGA)

Thrombin generation was measured by means of the Calibrated Automated Thrombography (CAT) method using a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland). Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) was used to calculate thrombin generation curves, from which four parameters were derived: lag time (initiation phase of coagulation), endogenous thrombin potential (ETP, area under the thrombin generation curve), peak height (maximal reaction) and time to peak (33). In this study, only the lag time and the ETP are reported as representative parameters. To 80 µL of plasma sample, 20 µL of trigger reagent (see below) were added and thrombin generation recording was started upon subsequent addition of 20 µL FluCa, a mixture of calcium chloride ('Ca', 87 mM) and thrombin substrate (Z-Gly-Gly-Arg-AMC, 2.5 mM , Bachem, Weil-am-Rein, Germany). Three different analytical conditions were applied:

First, a contact activator ('S', Synthasil, Instrumentation Laboratory, Zaventem, Belgium, final concentration 1/400) was added to trigger the intrinsic coagulation pathway, and 'lag time(Ca,S)' and 'ETP(Ca,S)' were recorded.

Second, tissue factor ('TF', Innovin, Siemens, Hamburg, Germany, final concentration 5 pM) was added to trigger primarily the extrinsic coagulation pathway and 'lag time(TF)' and 'ETP(TF)' were recorded.

Third, thrombin generation was performed upon simple recalcification of microvesicle-rich plasma in the absence of an exogenous trigger, to investigate the effect of endogenous coagulation triggers in the plasma sample, including microvesicle-bound factors such as TF, and 'lag time(Ca)' and 'ETP(Ca)' were recorded.

In TGA, especially the lag time is to a large extent determined by the amount of TF present in the assay (34). Hence, to functionally asses the endogenous TF in the plasma sample, we additionally measured 'lag time(Ca)' in the presence of 300 ng/mL (final concentration) tissue factor pathway inhibitor (TFPI, R&D Systems, Abingdon, UK). This enabled us to specifically investigate the TF-dependency of associations with air pollution exposure, as explained in detail in the data supplement.

All TGA's were performed in the presence of an excess (4 µM) of exogenous phospholipids (phosphatidylserine 30% and phosphatidylcholine 70%, Sigma-Aldrich, Bornem, Belgium), making thrombin generation independent of the surface expression on microvesicles of negatively charged phospholipids.

Microvesicle analysis by flow cytometry

Microvesicles were analyzed by flow cytometry following a protocol, standardized by the Scientific and Standardization Subcommittee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH)(35) with some adaptations. In brief, to thawed microvesicle-rich plasma, fluorescein isothiocyanate-labeled mouse anti-CD42a (BD Biosciences, Erembodegem, Belgium), phycoerythrin-labeled mouse anti-glycophorin A (GPA, BD Biosciences, Erembodegem, Belgium) and allophycocyanin-labeled annexin V (AV, Immunotools, Friesoythe, Germany) were added and samples were analyzed on a FACSCantoII flow cytometer (BD Biosciences, Erembodegem, Belgium) to define blood platelet-derived microvesicles ('BPμV', CD42a+), red blood cell-derived microvesicles ('RBCμV', GPA+) and microvesicles with a procoagulant, negatively charged phospholipid surface, binding annexin V ('AV^{+}μV'). A detailed description of our microvesicle analysis by flow cytometry is provided in the data supplement.

Tissue factor mRNA in circulating white blood cells

TF mRNA expression measurement was performed by quantitative real-time PCR on the AB 7500 Fast PCR System (Applied Biosystems, Ottignies-Louvain-la-Neuve, Belgium) as described in the data supplement.

Statistical analysis

For database management and statistical analysis, we used SAS Software (version 9.1, SAS Institute Inc, Cary, NC). Non-normally distributed data were log transformed. We investigated associations between plasma markers and recent or chronic exposure parameters using multiple linear regression. In all regression models we included the following a priori chosen covariates: gender, age, body-mass index, socioeconomic status, type of diabetes, physical activity, blood glucose levels, use of insulin, use of statins, use of antiplatelet medication and temperature and humidity on the day of blood sampling. Potential interactions of type of diabetes, use of statins and use of antiplatelet medication with the association between air pollution and all measured parameters were investigated. Q-Q plots of the residuals were used to test the assumptions of all linear models.

Results

Characteristics of the study population

Characteristics of study participants are shown in table 1. Of the men, 34 (32%) had type 1 diabetes compared to 57 (45%) for the women. All patients with type 1 diabetes used insulin; among type 2 diabetics, 126 persons (89%) used insulin medication. Mean values ±SD for HbA1c were 7.8±1.1% for type 1 diabetics and 7.2±1.2% for type 2 diabetics. Exposure characteristics are shown in table 2.

A representation of the location of the patients' residences and of the study center is shown in Fig. 2.

Table 1: Population characteristics

	Mean (SD) or number (%)
Gender, men	107 (46%)
Age, years	57.9 (17.5)
BMI, kg/m²	28.9 (5.5)
Type 1 diabetes	91 (39%)
Exposure to environmental tobacco smoke	34 (15%)
Socioeconomic status	
Low	158 (68%)
Middle	55 (23.5%)
High	17 (7.5%)
Unknown	3 (1%)
Antiplatelet medication[a]	140 (60%)
Statin	154 (66%)
ACE inhibitor	129 (55%)
Insulin	217 (93%)
Oral antidiabetic medication	106 (46%)
Blood glucose, mg/dL	139.2 (63.4)[b,c,d]
Glycated hemoglobin, %	7.5 (1.2)[e,f]

[a]Antiplatelet medication includes acetylsalicylic acid, clopidogrel, ticlopidine or dipyridamole
[b]Non-fasting values
[c]Data are available for 224 persons
[d]Reference values (fasting): 55-100 mg/dL
[e]Data are available for 229 persons
[f]Reference values: 4.0-6.0 %

Clinical measurements

The value distribution of clinical parameters for the study population is shown in table S1 (data supplement). With the exception of FVIII, mean values for the population were within the normal reference ranges. Figure 3 shows the associations (adjusted for the aforementioned covariates) between air pollution exposure and all outcome parameters, as determined by the regression analysis for different time windows. The corresponding effect sizes for selected time windows are shown in figures 4 to 6. A stratified analysis for type of diabetes, statin use or antiplatelet use is shown in supplementary figures S5-7 (data supplement).

Inflammation parameters and blood cells

Significant positive correlations were observed between PM_{10} exposure at the patient's residence and hsCRP and WBC concentrations for PM_{10} exposure windows within 1 week (Fig. 3), with positive but only borderline significant values ($0.05<p<0.10$) for the longer time windows up to 6 months. Each 10 $\mu g/m^3$ increase

in the mean PM_{10} concentration over the preceding week at the patient's residence increased
the hsCRP by 23% (95%CI: 5-45) and the WBC by 7% (95%CI: 2-12) (Fig. 5A).

Table 2: Exposure characteristics of included subjects (n=233).

Type of exposure	mean	median	range	P10-P90	IQR
Current indoor $PM_{2.5}$, $\mu g/m^3$	4.6	4.3	1.4-7.6	2.4-7.2	2.6
Current indoor PM_{10}, $\mu g/m^3$	22.1	21.3	12.0-39.4	15.0-30.8	5.6
Residential PM_{10}, $\mu g/m^3$					
day 0	25.7	26.3	7.3-52.8	10.7-36.2	11.4
day -1	27.6	28.0	7.2-55.7	13.0-40.9	11.5
day -2	28.0	27.4	9.9-85.8	14.2-42.5	11.4
day -3	28.3	26.5	7.7-72.6	13.1-47.5	16.2
mean 1 week	25.2	25.8	11.8-44.4	14.2-37.8	16.5
mean 1 month	26.3	25.5	11.6-43.0	19.9-34.3	8.5
mean 3 months	25.9	25.7	15.0-38.3	22.9-29.2	3.08
mean 6 months	22.8	22.6	13.3-34.6	20.4-25.5	2.7
mean 1 year	22.1	21.7	12.5-33.7	19.3-25.07	2.7
Residential distance to major road, m	689	410	10-5191	10-1767	755

IQR: interquartile range, P10: percentile 10, P90: percentile 90.

"Traditional" coagulation parameters

Current PM_{10} exposure correlated with a prolongation of the PT (Fig. 3). No significant correlations were found between either *current, subacute, subchronic* or *chronic* PM exposure and measurements of aPTT, FVII, FVIII, FXII or D-dimers (Fig. 3). Concentrations of fibrinogen correlated positively with PM_{10} at 'day-2' and 'day-3', as well as with the mean PM_{10} concentration over 1 week (Fig. 3). Each 10 $\mu g/m^3$ increase in the mean concentration of PM_{10} over the preceding week at the patient's residence elevated fibrinogen levels by 4% (95%CI: 1-7) (Fig. 5A). Each halving in residential distance to a major road increased FVIII by 2% (95%CI: 1-3) (Fig. 6).

Thrombin generation

In contrast to the paucity of relationships between PM exposure and the above mentioned hemostasis parameters, we found strong significant correlations with various parameters of thrombin generation over many different time windows of exposure (Fig. 3).

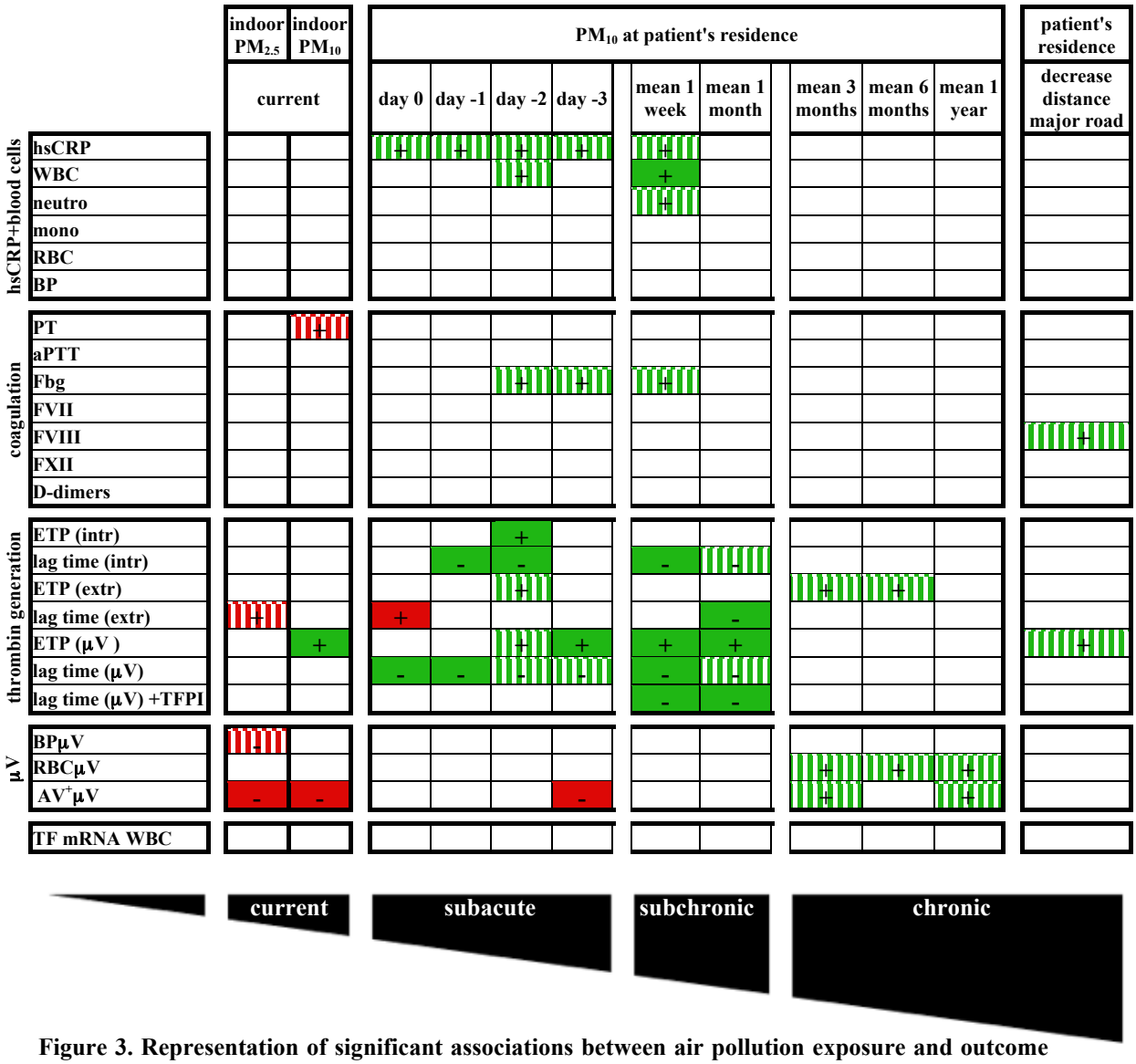

Figure 3. Representation of significant associations between air pollution exposure and outcome parameters

Regression analysis was performed to determine the associations between outcome parameters and current $PM_{2.5}$ and PM_{10} concentrations measured in the diabetes outpatient clinic's waiting room, residential PM_{10} concentrations over different time windows preceding blood sampling (as indicated) or residential distance to a major road (N- or E-road). For significant associations, positive and negative slopes are denoted as '+' or '-' respectively. Significant associations that represent increases in inflammatory parameters or blood cells, or procoagulant changes, are highlighted in green striped ($p<0.05$) or green plain ($p<0.005$) boxes. Significant associations that represent decreases in inflammatory parameters or blood cells, or anticoagulant changes, are highlighted in red striped ($p<0.05$) or red plain ($p<0.005$) boxes.

The strongest correlations were found for the lag time during thrombin generation with a contact activator ['lag time(Ca,S)'] and for both the lag time and ETP after simple recalcification of microvesicle-rich plasma, in the presence of standardized phospholipid concentrations ['lag time(Ca)' and 'ETP(Ca)']. These significant correlations were consistent for the *subacute* and *subchronic* time windows, with the

highest effect sizes measured for the mean PM_{10} concentration over 1 month: each 10 $\mu g/m^3$ increase in PM_{10} shortened the 'lag time(Ca,S)' by 6% (95%CI: 0-12, p=0.08), the 'lag time(Ca)' by 14% (95%CI: 4-25) and increased the 'ETP(Ca)' by 14% (95%CI: 5-24). Interestingly, upon the addition of a specific tissue factor inhibitor (TFPI) to the assay, the positive associations between PM_{10} and 'lag time(Ca)' disappeared for the subacute windows, but not for the subchronic time windows (Fig. 3). Each halving in residential distance to a major road increased the 'ETP(Ca)' by 2% (95%CI: 0-4) (Fig. 6)

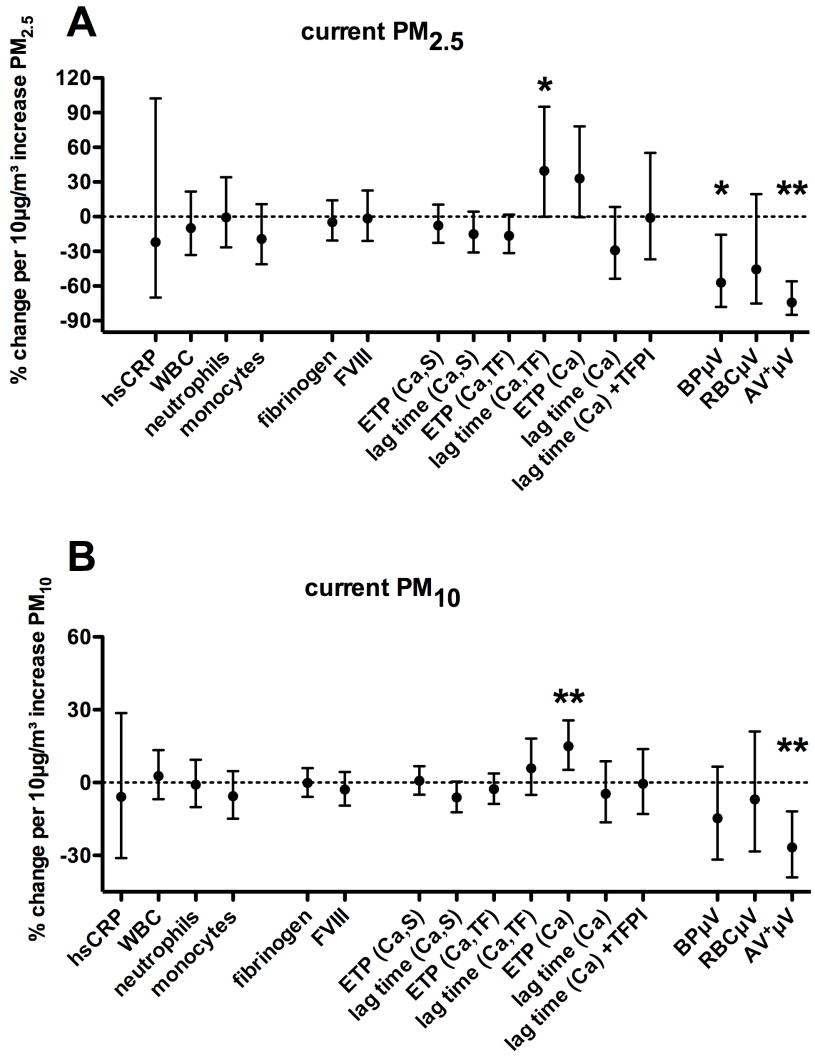

Figure 4. Effect sizes for significant associations with current $PM_{2.5}$ and PM_{10} concentrations
Effect sizes (%, 95%CI) were calculated (A) for each 10 $\mu g/m^3$ increase in current $PM_{2.5}$ and PM_{10} concentrations, measured in the diabetes outpatient clinic's waiting room in the hours preceding the blood sampling. Those parameters were selected that showed significant correlations in figure 3. hsCRP=high sensitivity CRP. WBC=white blood cells, RBC=red blood cells, BP=blood platelets, ETP=endogenous thrombin potential, BPµV=BP-derived microvesicles, RBCµV=RBC-derived microvesicles, $AV^+\mu V$=annexin-V binding microvesicles. Analysis adjusted for covariates.

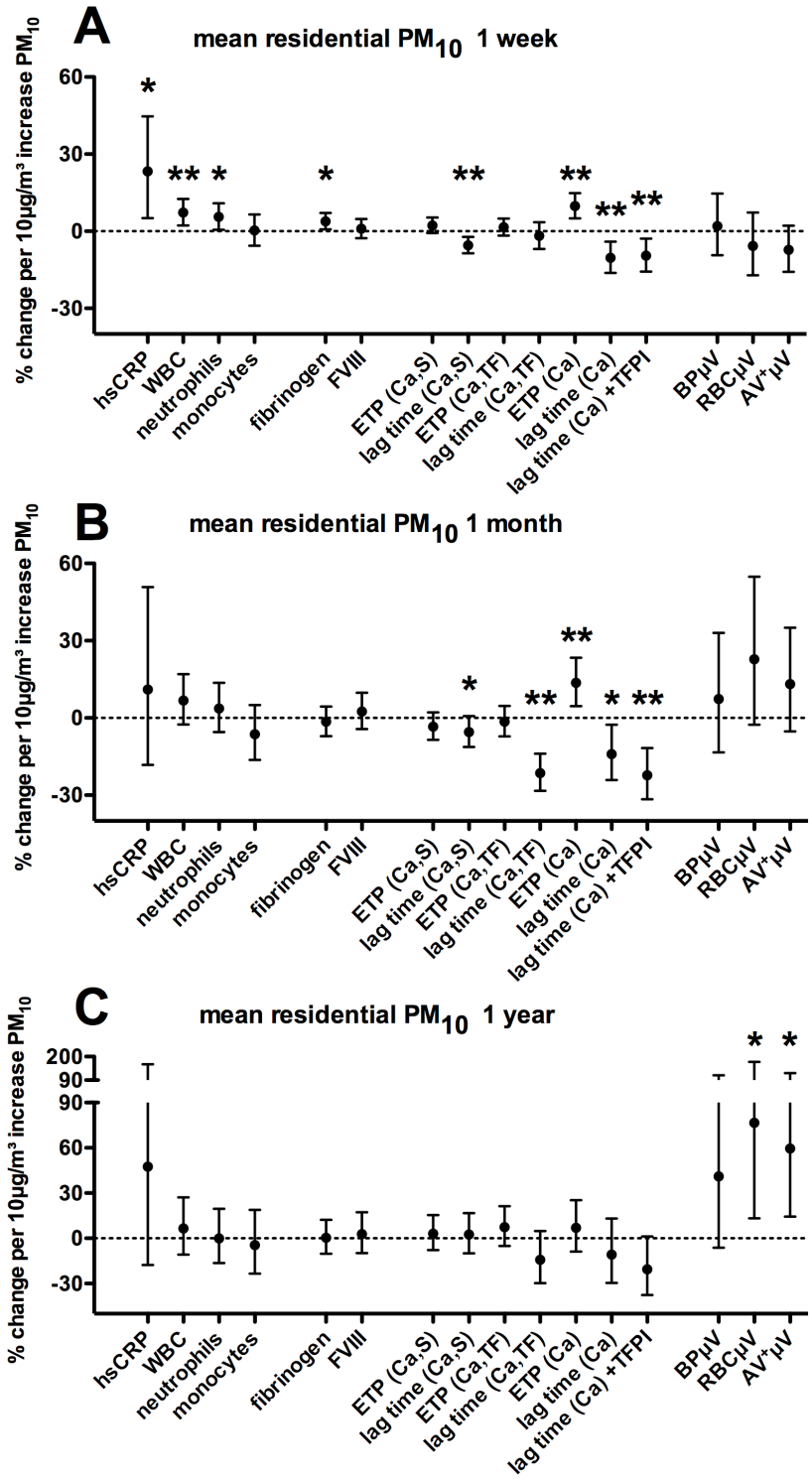

Figure 5. Effect sizes for significant associations with residential PM_{10} concentrations at different time windows before blood sampling

Effect sizes (%, 95%CI) were calculated for each 10 µg/m^3 increase in residential mean PM_{10} concentrations measured over (A) 1 week, (B) 1 month or (C) 1 year preceding the blood sampling. Those parameters were selected that showed significant correlations in figure 3. hsCRP=high sensitivity CRP. WBC=white blood cells, RBC=red blood cells, BP=blood platelets, ETP=endogenous thrombin potential, BPµV=BP-derived microvesicles, RBCµV=RBC-derived microvesicles, AV^+µV=annexin-V binding microvesicles. Analysis adjusted for covariates.

Microvesicle analysis

The concentrations of *current* $PM_{2.5}$ correlated negatively with the concentrations of BPμV (-57%, 95%CI: -78 to -16% per 10 μg/m^3 increase in $PM_{2.5}$) and of AV^+μV that express negatively charged phospholipids on their surface (-74%, 95%CI: -85 to -56% per 10 μg/m^3 increase in $PM_{2.5}$), while both *current* PM_{10} and residential PM_{10} on 'day -3' correlated negatively with the concentration of AV^+μV (-27%, 95%CI: -39 to -12% per 10 μg/m^3 increase in current PM_{10}) (Fig. 3 and 4). In contrast, we found positive correlations between mean PM_{10} concentrations over the *chronic* exposure windows (preceding 3 months to 1 year) and the concentrations of RBCμV and AV^+μV with the highest effect sizes measured for the 1-year period (+77% 95%CI: 14-176 and +60% 95%CI: 15-123 per 10 μg/m^3 increase in yearly PM_{10}, respectively) (Fig. 5C). Similar borderline significant results were found for the BPμV.

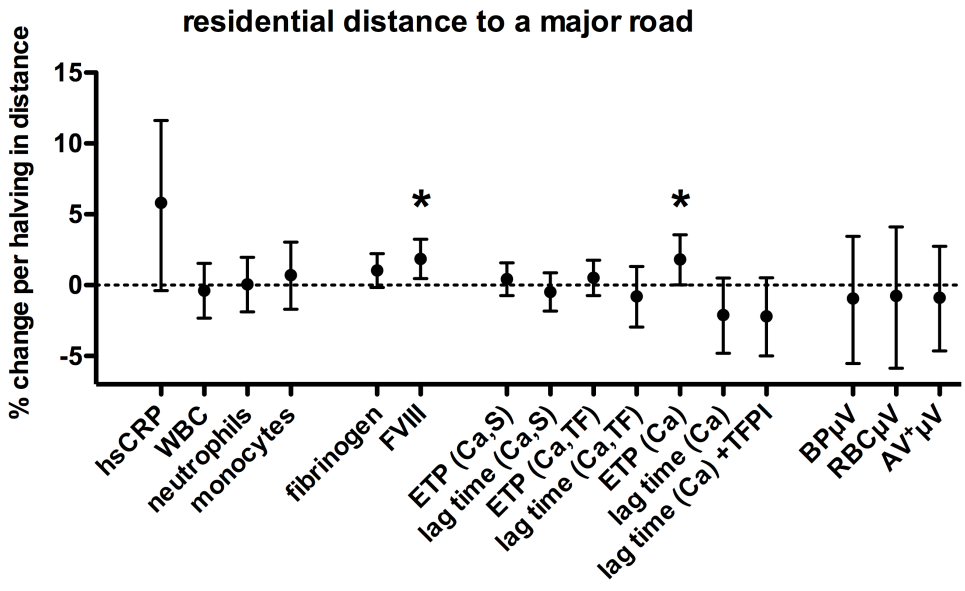

Figure 6. Effect sizes for significant associations with residential distance to a major road
Effect sizes (%, 95%CI) were calculated for each halving in distance from the patient's residence to the nearest major road (N- or E-road). Those parameters were selected that showed significant correlations in figure 3. hsCRP=high sensitivity CRP. WBC=white blood cells, RBC=red blood cells, BP=blood platelets, ETP=endogenous thrombin potential, BPμV=BP-derived microvesicles, RBCμV=RBC-derived microvesicles, AV^+μV=annexin-V binding microvesicles. Analysis adjusted for covariates.

TF mRNA in circulating WBC

No significant correlations were observed between air pollution exposure and levels of TF mRNA expression in circulating white blood cells (Fig. 3).

Effect-modification

Table 1 demonstrates, for our study population of people with diabetes, the frequent use of different types of medication, some of which (e.g. statins) have been described to influence levels of TF or microvesicles. Therefore, we investigated if type of diabetes, use of statin medication and use of antiplatelet medication induced effect-modification on the association between PM exposure and outcome variables. Results are shown in figures S5-7 of the online data supplement.

In general, stratification for type of diabetes (Fig. S5) or medication (Fig. S6-7) did not considerably influence the non-stratified associations described in figure 3. For patients with type 1 diabetes, increased current PM exposure was associated with decreases in inflammatory parameters (hsCRP, fibrinogen), in thrombin generation ['ETP(Ca,S)' and 'ETP(Ca,TF)'] and microvesicles (BPμV, RBCμV, AV^{+}μV) that were not, or to a lesser extent, observed in type 2 diabetics, who rather showed increases in inflammatory parameters (Fig. S5), compatible with the more inflammatory nature of this type of diabetes. Both statins (Fig. S6) and antiplatelet use (Fig. S7) tended to decrease the effect size of associations between chronic (1 week-1 year) PM exposure and inflammatory (WBC, neutrophils) or procoagulant ['ETP(Ca,S)' and 'ETP(Ca,TF)'] changes, as compared to patients not taking these medications.

Discussion

Parameters of inflammation, coagulation and microvesicles were correlated with measures of *current* (at blood sampling), *subacute* (day 0 to day -3), *subchronic* (mean 1 week to 1 month) and *chronic* (mean 3 months to 1 year) PM exposure and with residential distance to a major road, in patients with diabetes. Especially type 2 diabetes is a chronic inflammatory disease and circulating microvesicles seem to be elevated in these patients (36, 37). It was, therefore, relevant to measure inflammation parameters and microvesicle numbers in patients with diabetes who manifest increased susceptibility to air pollution (29).

Current PM levels were associated with lower numbers of circulating microvesicles and with decreased inflammatory parameters, mainly in patients with type 1 diabetes.

We even found an isolated prolongation of the PT with increased levels of current PM_{10}, contrasting previous observations (14, 38). However, the lower number of circulating microvesicles should not necessarily be interpreted as an anti-inflammatory response to short-term PM exposure but can be explained by the recruitment of circulating microvesicles to the lung via enhanced expression of adhesive receptors, including P-selectin (39, 40) and von Willebrand factor (41), by acutely activated pulmonary endothelial cells.

In contrast to the *current* PM exposure, consistent proinflammatory and procoagulant changes were observed for the longer PM exposure windows.
Subacute and *subchronic* exposure up to 1 week were associated with a systemic inflammatory status, evidenced by increased hsCRP, total WBC counts and neutrophil counts. In agreement with other studies (15, 42, 43), and compatible with its role as an acute phase protein, fibrinogen concentrations increased with higher PM exposure levels, within 1 week. We did not measure increases in FVIII, another acute phase protein, but the high mean baseline value for FVIII in a diabetic study population (table S1 and (44)), could hinder further increase by PM exposure.
Yet, increased fibrinogen concentrations cannot explain the strong correlations observed here between different thrombin generation parameters and *subacute* and *subchronic* PM_{10} exposure up to 1 month, since TGA are not influenced by fibrin(ogen) levels. Likewise, in the absence of procoagulant changes in any of the other "traditional" coagulation parameters (PT, aPTT, FVII, FVIII, FXII, D-dimers), other processes should be responsible for enhancing thrombin generation with higher levels of PM exposure so consistently.

A role for microvesicles, cellular bodies released from stimulated or apoptotic cells, in VTE has been suggested (27, 45). We assessed the procoagulant potential of microvesicles through measurement of their surface expression of TF in TGA, and through the analysis of the number of red blood cell and blood platelet-derived microvesicles via flow cytometry. In addition, flow cytometric analysis of annexin V-binding was undertaken to measure the surface expression of negatively charged phospholipids (mainly phosphatidylserine) (26). Specifically the latter measurement has one drawback, i.e. that freezing-thawing affects the expression of negatively charged phospholipids (46, 47), and therefore may not provide an accurate index of

phosphatidylserine exposure *in vivo*. However, the consistency of the strong associations of the number of $AV^+\mu V$ with PM exposure over the different longer time windows indicates that these associations are unlikely to be artificially induced chance findings.

Both *subacute* and *subchronic* PM exposure correlated strongly (p values <0.0001) with thrombin generation. The most pronounced correlations were found for TGA performed in the absence of an external trigger of coagulation ('lag time(Ca)' and 'ETP(Ca)'), and may therefore depend on the presence of endogenous triggers present in the plasma, such as contact activation or microvesicle-bound TF, the concentration of the latter having a pronounced effect on the lag time (34).
In the *subacute* time window, associations between PM_{10} and lag time disappeared both in the presence of an excess of exogenous TF ['lag time(Ca,TF)'] and upon inhibition of TF by TFPI ['lag time(Ca)+TFPI']. This points towards exposure-associated increased levels of circulating TF, most likely on microvesicles (48). The source of TF-bearing microvesicles is a matter of debate, yet with activated monocytes being the most likely candidate (49). The remarkable conincidence in the subacute time window of associations of PM exosure with inflammatory parameters and with TF-dependent changes in TGA adds to the hypothesis of inflammation-coagulation cross-talk in the first days following exposure to air pollution. In healthy individuals, microvesicles derived from blood platelets and red blood cells account for almost all circulating microvesicles. Since WBC-derived microvesicles are extremely low (50), we could not quantify these microvesicles via flow cytometry.

In the *subchronic* time window, assocations between PM exposure and thrombin generation were no longer dependent on TF. Associations with lag times were also present upon exogenous TF addition ['lag time(Ca,TF)'] and did not disappear upon addition of TFPI. Moreover, procoagulant changes at 1 month occured in the absence of those inflammatory changes mentioned above. Therefore, the enhanced thrombin generation associated with PM concentrations over several weeks must be explained by (an) other mechanism(s). A recent study in mice suggests that PM promotes early procoagulant changes mostly through a TF-driven extrinsic pathway of coagulation, whereas long lasting procoagulant effects are predominantly mediated through contact activation of the intrinsic pathway of coagulation by systemically translocated ultra-

fine particles (51). Yet, we found no associations between FXII and exposure to PM. An alternative explanation could be offered by downregulation of the anticoagulant pathways, including protein C and antithrombin, but these markers were not assessed in the present study.

In the *chronic* PM exposure window, procoagulant changes were no longer obvious from thrombin generation measurements. Yet, a procoagulant tendency was apparent from the higher microvesicle numbers, both blood-platelet derived and red blood cell-derived, and increased microvesicular annexin V binding, reflecting surface expression of negatively charged phospholipids (mainly phosphatidylserine) (26). TGA in the present study was performed in the presence of an excess of exogenous phospholipids, and is therefore insensitive to the effects by endogenous negatively charged phospholipids. We found the highest effect size on microvesicle number and procoagulant potential for the mean PM_{10} measurement over 1 year. Interestingly, in the study by Baccarelli et al. (9), a 1-year exposure time window correlated most strongly with the risk of DVT, while no significant correlations were found for time windows shorter than 9 months. Hence, upregulation of procoagulant microvesicles could, at least partly, be a pathophysiological mechanism underlying the association between long-term PM exposure and VTE (9, 27, 28).

Living near a *major road* has been associated with increased risk for VTE in a case-control study (10). A recent population-based prospective cohort study (13), also demonstrated an, admittedly, nonsignificant 16% increase in the risk of VTE for subjects living within 150 m of a major traffic road. In the present study, the correlations with residential *distance to a major road* were, although following similar trends, fewer and weaker than for the *chronic* residential PM_{10} measurements by the land-use interpolation model. This is further discussed in the online data supplement.

Our study has limitations. First, association studies do not prove causality, and our observations can therefore only suggest that procoagulant changes, consisting of enhanced thrombin generation and higher numbers of procoagulant microvesicles, are induced by exposure to PM. Second, the “disappearance” of associations between subacute PM exposure and thrombin generation in the presence of TFPI in the assays

demonstrates a crucial role for circulating TF, which is not necessarily all microvesicle-bound. Indeed, TF in plasma is primarily located on microvesicles, but it can also circulate as an alternatively spliced soluble protein (48). Nevertheless, plasma depletion of microvesicles by filtration through a 0.1 μm filter significantly prolonged the lag time of thrombin generation assays in previous experiments in our laboratory (data not shown), suggesting that microvesicles are indeed the major source for TF. Third, a large number of statistical analyses was performed in this study, increasing the possibility of rejecting the null hypothesis too readily. However, correction for multiple testing is not always appropriate, as discussed in larger detail in the online data supplement.

In conclusion, this study demonstrates for the first time that increases in the number and the procoagulant potential of microvesicles, rather than increases in coagulation factors per se, may contribute to the prothrombotic risk induced by air pollution exposure.

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Data supplement

Supplemental Methods and Results

Distribution of clinical parameters

Mean values and standard deviation of clinical parameters for the study population are shown in table S1, together with the reference ranges for the routine clinical parameters.

Table S1: distribution of clinical parameters for the whole study population and reference ranges

	mean	SD	reference range
hsCRP (mg/L)	2.8	3.6	<5.0
WBC (10^3/μL)	7.3	2.0	3.1-8.9
neutro (10^3/μL)	4.6	1.5	0.5-5.9
mono (10^3/μL)	0.6	0.2	0.3-0.7
RBC (10^6/μL)	4.5	0.5	m: 4.5-6.0 f: 3.9-5.6
BP (10^3/μL)	294	85	150-450
PT (sec)	8.9	1.5	7.0-9.7
aPTT (sec)	26.2	2.5	23.3-34.5
Fbg (g/L)	3.4	0.8	0.9-3.8
FVII (%)	113	30	70-130
FVIII (%)	187	52	70-150
FXII (%)	100	18	70-150
D-dimers (mg/L)	0.470	0.481	0.000-0.490
ETP(Ca,S) (nM.min)	1436	300	
lag time(Ca,S) (min)	8.7	2.2	
ETP(Ca,TF) (nM.min)	1525	346	
lag time(Ca,S) (min)	2.6	1.4	
ETP(Ca) (nM.min)	1387	325	
lag time(Ca) (min)	25.4	12.4	
lag time(Ca)+TFPI (min)	31.4	13.8	
BPμV (count)	2272	1604	
RBCμV (count)	597	1076	
AV^+μV (count)	1614	949	
TF mRNA WBC (copies/10^6 copies GaPDH)	1458	15129	

m: male, f: female

Introduction to the analysis of microvesicles

Figure S1 gives a schematic representation of the role of microvesicles in the physiology of intravascular coagulation and specifies the assays used in the present study to measure the microvesicles' procoagulant potential. The procoagulant potential of microvesicles (μV) relates to the surface expression of tissue factor (TF), the main coagulation trigger *in vivo*, and of negatively charged phospholipids (PL^-), to which different activated coagulation factors bind in a Ca^{2+}-dependent manner to support the coagulation cascade, resulting in the formation of a fibrin clot. The quantification of blood platelet-derived and red blood cell-derived microvesicles was performed by flow cytometry. The procoagulant potential of microvesicles was evaluated through measurement of their surface expression of tissue factor (TF) by thrombin generation assays (TGA) in a phospholipid-independent way, and through measurement of their surface expression of negatively charged phospholipids (mainly phosphatidylserine) by a TF-independent flow cytometric analysis of annexin V binding.

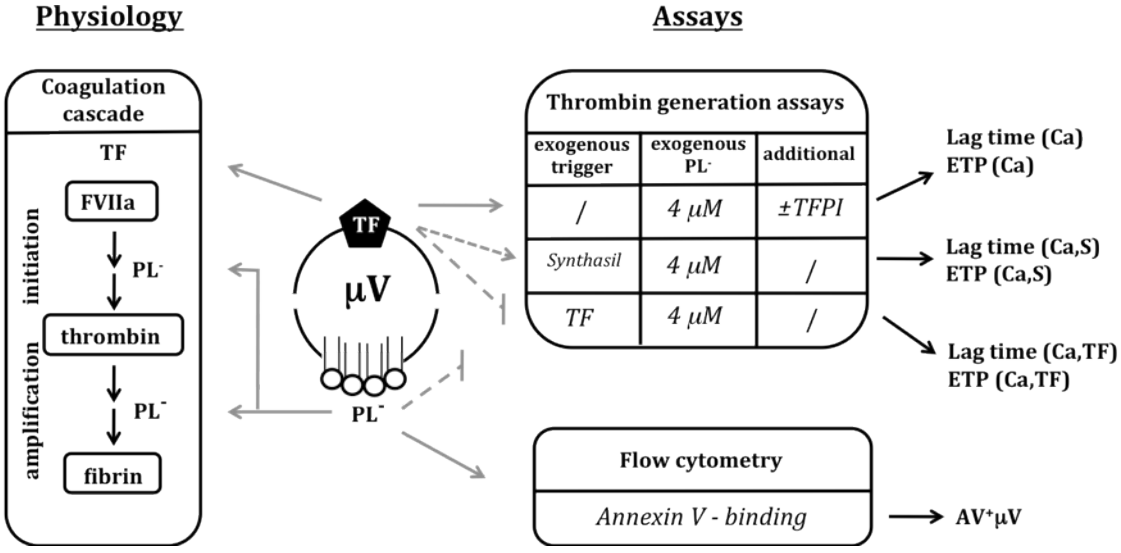

Figure S1. Microvesicles' procoagulant potential

The left side of the figure shows a simplified scheme of the coagulation cascade. The right side of the figures shows the different assays used in this study to measure the microvesicles' procoagulant potential. TF, present on the microvesicles' surface, largely determines 'lag time(Ca)', 'endogenous thrombin potential ETP(Ca)', and to a lesser extent 'lag time(Ca,S)' and 'ETP(Ca,S)'. It does not influence extrinsic parameters, due to the addition of exogenous TF in the assay. The presence of PL^- on the microvesicles' surface is measured through the binding of annexin V (AV) in a flow cytometric assay. These PL^- do not influence TGA's since an excess (4 μM) of exogenous PL^- is added in these assays. TFPI=tissue factor pathway inhibitor.

Thrombin generation assays

Thrombin generation assays (TGA) were performed as described in the main article. In TGA, lag times are to a large extent determined by the amount of TF present in the assay (1). Hence, to determine the contribution to thrombin generation of endogenous TF, present in the plasma sample, 'lag time(Ca)' was measured as described in the main article, both in the presence and the absence of 300 ng/mL (final concentration) tissue factor pathway inhibitor (TFPI, R&D Systems, Abingdon, UK). This enabled us to specifically investigate the TF-dependency of associations with PM exposure.

Figure S2A shows how increasing concentrations of TF dose-dependently shorten the lag time of thrombin generation. Figure S2B demonstrates how increasing concentrations of TF pathway inhibitor (TFPI) prolong the lag time to result in a complete inhibition of exogenously added TF (0.5 pM) -induced thrombin generation, at a concentration of 300 ng/mL TFPI. Based on these initial experiments, a dose of 300 ng/mL was chosen to inhibit endogenous TF in our experiments. This concentration was deemed high enough in our patient samples, which all showed lag times over 10 minutes, i.e. at best contained 0.5 pM TF (Fig. S2A).

A representative example for this analysis in a patient sample is shown in figure S2C. The remaining, but retarded thrombin generation in the presence of TFPI, reflects TF-independent coagulation activation, induced by contact activation. This part can be inhibited in the presence of corn trypsin inhibitor (not shown).

Microvesicle analysis by flow cytometry

Microvesicles were analyzed by flow cytometry following a protocol, standardized by the Scientific and Standardization Subcommittee (SSC) International Society on Thrombosis and Haemostasis (ISTH) (2, 3) with some adaptations. Blood was collected on 3.8% citrate, and centrifuged twice at 1900 x g (10 min. and 20 min. respectively), within one hour after sampling, to obtain blood platelet-depleted and microvesicle-rich plasma. Subsequently, samples were immediately stored at -80°C for future analysis. Microvesicle-rich plasma samples were thawed for 20 minutes at 37°C. To 20 μL of plasma, 55 μL of buffer (Hepes 10mM, NaCl 140 mM, CaCl2 2.5 mM, pH 7.4) containing hirudin (55 μg/mL Refludan, Celgene, Braine-l'Alleud, Belgium), 10 μL of fluorescein isothiocyanate-labeled mouse anti-CD42a (BD Biosciences, Erembodegem, Belgium), 5 μL of phycoerythrin-labelled mouse anti-

glycophorein A (GPA, BD Biosciences, Erembodegem, Belgium) and 10 µL of allophycocyanin-labelled annexin V (AV, Immunotools, Friesoythe, Germany) were added.

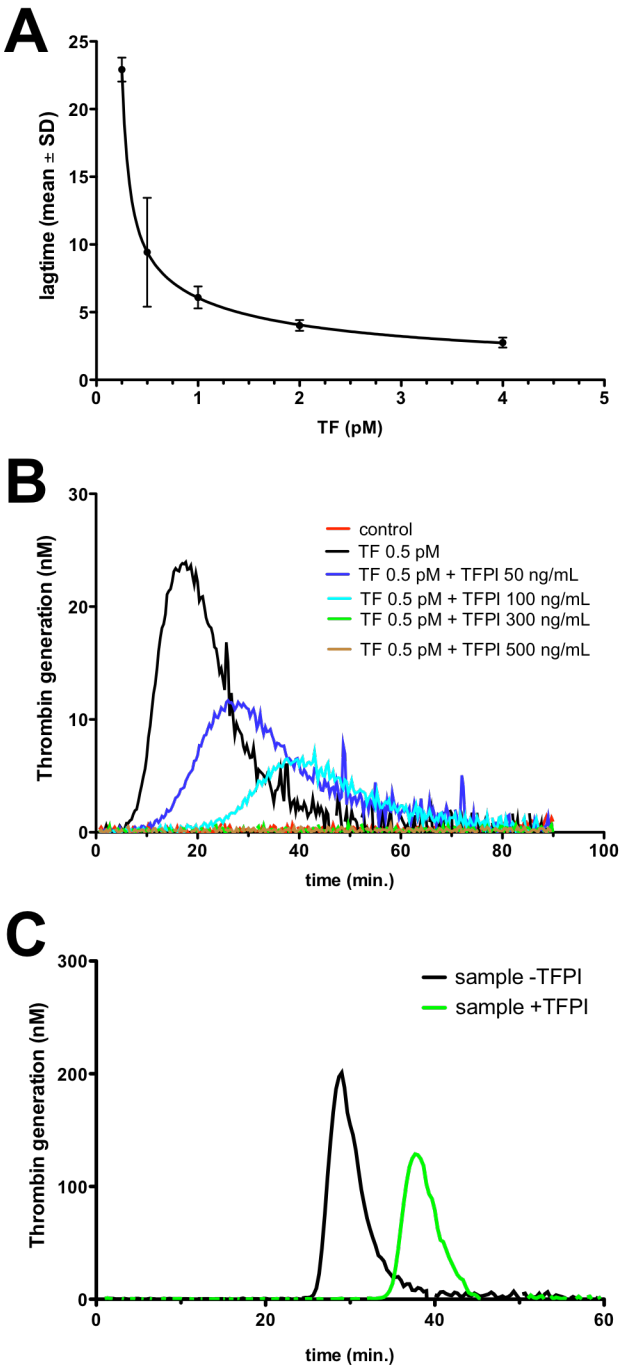

Figure S2: correlation between tissue factor concentration and lag time.
(A and B) Human plasma was collected on 3.8% citrate containing corn trypsin inhibitor (CTI, final concentration 10 µg/mL) to prevent contact activation. Plasma was centrifuged twice at high speed to lower the number of microvesicles. All assays were performed in duplicate in 3 independent experiments in the presence of 4 µM exogenous phospholipids. (A) Exogenous tissue factor (TF, Innovin, Siemens) was added to human plasma at different final concentrations (0.25, 0.50, 1, 2 and 4 pM) and the lagtime was measured upon recalcification. (B) Plasma was spiked with 0.5 pM TF and with different concentrations of TFPI (0, 50, 100, 300 and 500 ng/mL). No thrombin generation was observed for the control condition, in which no TF nor TFPI were added. (C) Representative example of a plasma sample from the present study with added TFPI (300 ng/mL, as indicated), in the absence of CTI.

After 20 minutes incubation at room temperature in the dark on a rocking platform, samples were diluted to a final volume of 500 μL with buffer (Hepes 10mM, NaCl 140 mM, CaCl2 2.5 mM, pH 7.4). All buffers were filtered (Millex-VV 0.1 μm, Millipore, Brussels, Belgium) and antibodies were centrifuged (10 minutes at 14,000g) to remove debris and aggregates.

Samples (90 μL) were analyzed on a FACSCantoII flow cytometer (BD Biosciences, Erembodegem, Belgium) at a low flow rate (0.5 μL/sec). Events were gated according to size in a forward/side scatter dot plot. Subsequently, gated events were plotted on logarithmic scaled fluorescence channel 1, channel 2 and channel 3 dot plots. Blood platelet-derived microvesicles (BPμV) were identified as CD42a-positive events and red blood cell-derived microvesicles (RBCμV) as GPA-positive events. Microvesicles with a procoagulant, negatively charged phospholipid surface were identified by their binding to annexin V (AV^+μV). Using these 3 markers, 8 different microvesicle populations can be defined: total BPμV, AV^+ BPμV, AV^- BPμV, total RBCμV, AV^+ RBCμV, AV^-RBCμV, total AV^+μV and finally AV^+μV that are negative for CD42a and for GPA. In this study, only total BPμV, total RBCμV and total AV^+μV are reported to simplify representation of data. Measurement of microvesicles after filtration of platelet-poor plasma through a 0.1-μm filter (Millex-VV 0.1, Millipore, Brussels, Belgium) served as a negative control. Samples were run at a low flow rate over 3 minutes and results expressed as absolute counts.

Figure S3 shows the validation of the performance of our flow cytometer according to the standardization protocol (2, 3) using fluorescent calibrated sub-micrometer beads (Megamix, BioCytex, Marseille, France).

Our protocol differs from the SSC protocol on some points: first, we centrifuged the samples twice at 1900 x g (10 min. and 20 min. respectively), while the SSC protocol prescribes an intitial centrifugation for 15 min. at 1500 x g, followed by a second centrifugation for 2 min. at 13,000 x g. We found that our centrifugation protocol was more effective in eliminating blood platelets than the SSC protocol, when measured on a Cell Dyn 3500 automated cell counter (Abbott Diagnostics, Abbott Park, IL, USA) (data not shown). Second, our selection of antibodies (CD42a-FITC, GPA-PE

and AV-APC) differed from that of the SSC protocol (CD42a-PE and AV-FITC). However, these protocol differences are unlikely to influence the observed correlations between PM exposure and microvesicle counts by flow cytometry.

In agreement with the SSC protocol, we did not define any size-limiting gates based on the forward scatter (FSC), since identification of size using FSC challenges the sensitivity limit of flow cytometry (3). Moreover, size-related information derived from plastic beads (Megamix) and that derived from biological parameters (samples) are not comparable (2).

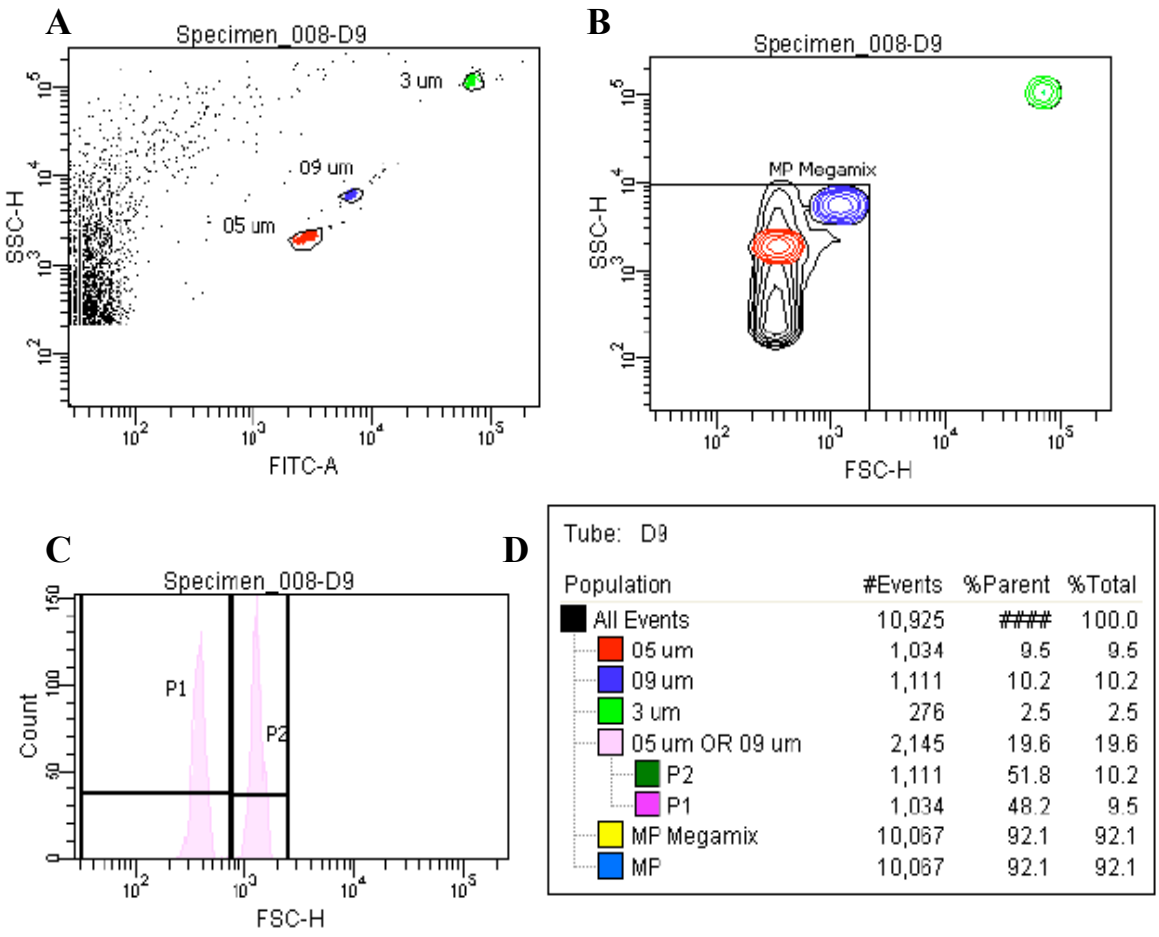

Figure S3: Validation of the BD FACSCantoII flow cytometer using Megamix beads. (A) On a FITC/side scatter plot, the 3 subsets of beads of differing diameters (0.5, 0.9 and 3 μm) can be distinguished. (B) A 'microvesicle gate' (MP, MP Megamix) was defined based on the position of the 0.9 μm beads subset (blue) in a forward/side scatter plot. (C) This histogram, showing the FSC distribution of the 0.5 ('P1') and 0.9 ('P2') μm beads, validates the resolution of our flow cytometer for microvesicle analysis. (D) Hierarchical description of populations and gates.

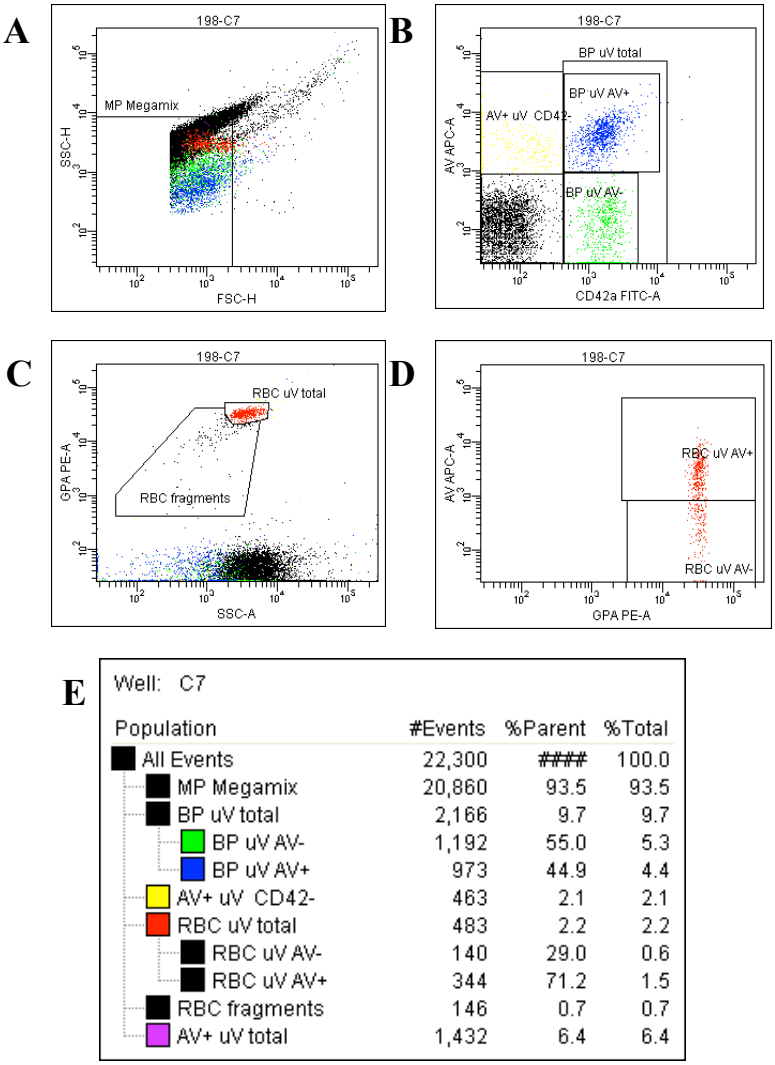

Figure S4: Representative example of flow cytometric microvesicle analysis in a sample. (A) Distribution of the different microvesicle populations in a forward/side scatter plot. Colors are backgated from the double fluorescence plots. (B) On a CD42a-FITC/AV-APC plot, the following populations are defined: BPμV that bind AV ('BP uV AV+', blue), BPμV that do not bind AV ('BP uV AV-', green) and microvesicles that bind AV but are negative for the BP marker CD42a ('AV+ uV CD42-', yellow). The combination of 'BP uV AV+' and 'AV+ uV CD42-' defines all microvesicles that bind AV ('AV+ uV total'). (C) On a side scatter/GPA-PE plot, the population of RBC-derived microvesicles ('RBC uV total', red) is defined. The events localizing at the left lower of this population are categorized as RBC fragments, and are not counted as microvesicles. (D) Only the 'RBC uV total' are plotted on this GPA-PE/AV-APC plot, to define RBCμV that bind AV ('RBC uV AV+') and RBCμV that do not bind AV ('RBC uV AV-'). (E) Hierarchical description of populations and gates.

A comparative study in 40 laboratories accounting for 59 flow cytometers, demonstrated that a size-limiting gate, based on e.g. the 0.9 μm beads present in the Megamix, is not applicable for most BD flow cytometers, since the localisation of the microvesicle populations can vary from being partly inside to completely outside this gate from one analyzer to another (2). The 'MP Megamix' gate, shown in figure S4, is

therefore only indicative and not restricting. Figure S4 shows a representative example of a sample analysis.

Tissue factor mRNA in circulating white blood cells.

Blood was collected in PAXgene Blood RNA tubes and mRNA extracted from the circulating white blood cells using the PAXgene Blood RNA kit (PreAnalytix, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Taqman Reverse Transcription Reagents (Applied Biosystems, Ottignies-Louvain-la-neuve, Belgium) were used for the reverse transcription and SYBR Green PCR mastermix (Applied Biosystems, Ottignies-Louvain-la-Neuve, Belgium) for quantitative real-time PCR in duplicate on the AB 7500 Fast PCR System (Applied Biosystems, Ottignies-Louvain-la-Neuve, Belgium) using the following primers: ccgaacagttaaccggaaga (TF, forward), tgcagtagctccaacagtgc (TF, reverse), tggtatcgtggaaggactcatgac (GaPDH, forward), atgccagtgagcttcccgttcagc (GaPDH, reverse). C_t values for tissue factor were expressed relatively to the C_t values for the house-keeping gene GaPDH.

Assesment of chronic air pollution exposure

In the present study, chronic air pollution exposure was assessed by estimation of the mean residential PM_{10} concentration over 3 months to 1 year, and by measurement of the residential distance to a major road. We found fewer and weaker correlations for clinical outcome parameters with residential *distance to a major road* than for the *chronic* residential PM_{10} measurements by the land-use interpolation model.

Distance to a major road is a measure of exposure to traffic-related air pollution, consisting of both gaseous pollutants and PM, mainly diesel exhaust particles (DEP) with a mean aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$). In contrast, the measurement of PM_{10} by the land-use interpolation model also includes the coarse fraction of PM (PM with a mean aerodynamic diameter between 2.5 and 10 μm) that derives predominantly from natural sources, especially crustal material. Important bioaerosols, such as endotoxin, pollen grains and fungal spores, are found mostly in the coarse fraction (and larger particles) (4), and might explain, at least partially, the differences in the results between the 2 exposure assessment techniques. As an alternative explanation, distance to major road as a measure for air pollution exposure

could be compromised by the density of the road network, the high degree of urbanization and industrial activity in our study area. In less populated or industrialized areas, distance to a major road would be the major determinant of individual air pollution exposure, but not so in a region with a high background level of PM.

Effect-modification

Potential interactions of type of diabetes and use of medication (statins and antiplatelet drugs) medication with the association between air pollution and all measured parameters were investigated. Figures S5-7 show the stratified effect sizes for the association between air pollution exposure and the different parameters, for patients with type 1 (black) vs type 2 (grey) diabetes (Fig. S5), for patients not taking (black) or taking (grey) statins (Fig. S6) and for patients not taking (black) or taking (grey) antiplatelet medication (Fig. S7). All analyses were corrected for gender, age, body-mass index, socioeconomic status, physical activity, blood glucose levels, use of insulin, temperature and humidity at the day of blood sampling, and type of diabetes (only S6 and S7), use of statins (only S5 and S7) and use of antiplatelet medication (only S5 and S6). Asteriks denote significant effect-modification ($p<0.05$ for interaction).

A large number of statistical analyses was performed in this study. Adjustment for multiple comparisons is usually recommended to avoid rejecting null hypotheses too readily. The theoretical basis for advocating routine adjustment for multiple comparisons is that chance serves as the first order explanation for observed phenomena. However, this hypothesis undermines one of the basic premises of epidemiological research, which holds that human biology follows regular laws that may be studied through observation of populations or patients. Moreover, if, as in our current study, outcome variables such as different parameters reflecting microvesicle function or coagulation factors are correlated, then correction for multiple testing is not indicated, because each new test does not provide a completely independent opportunity for a type I error. Under such circumstances, adjustment for multiple comparisons is inappropriate**.**

Supplemental References

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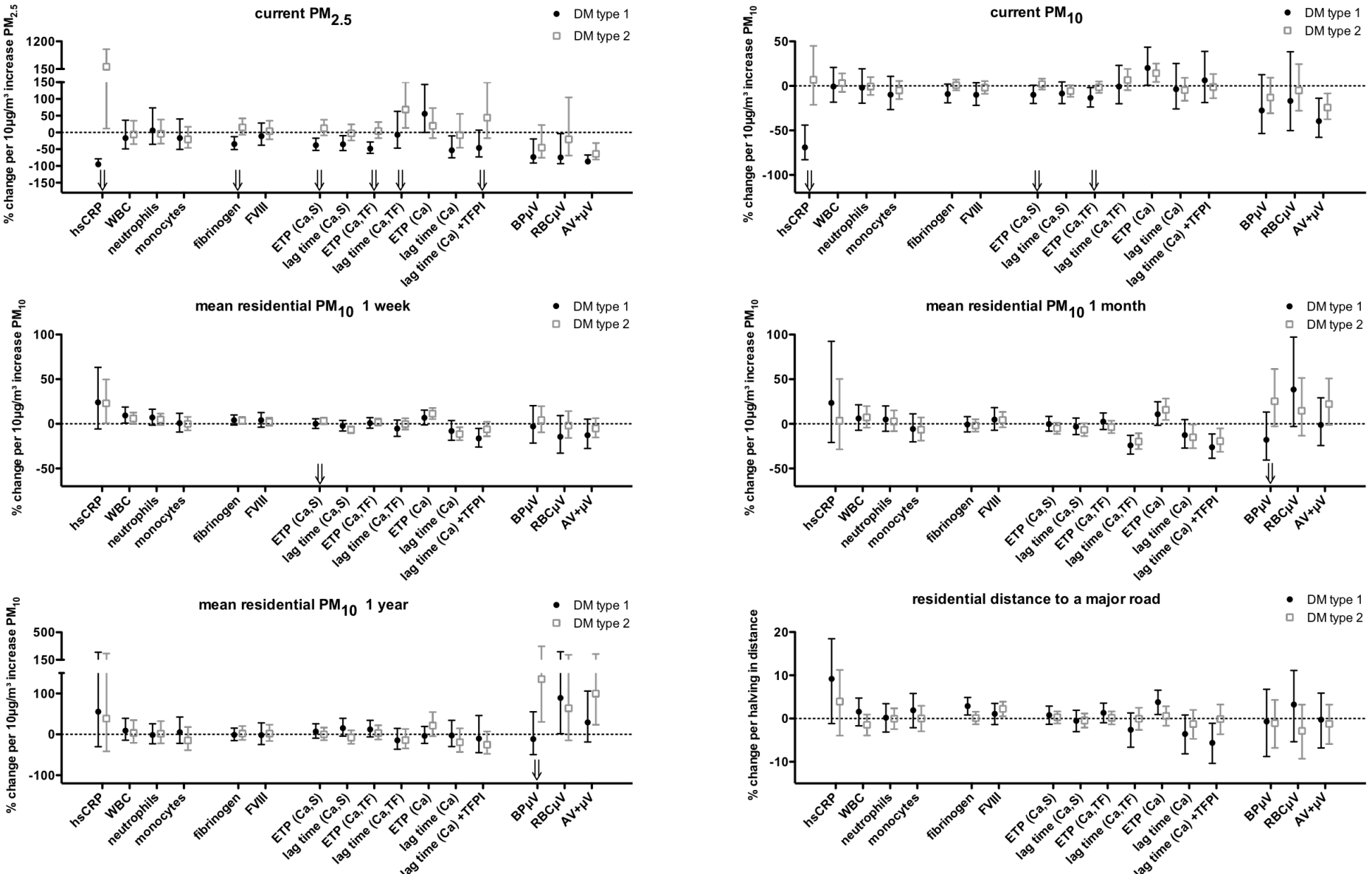

Figure S5: Effect modification for type of diabetes. Effect sizes for the association between air pollution exposure at different time windows (as indicated) and different parameters were stratified for patients with type 1 (black) vs type 2 (grey) diabetes. Analysis adjusted for covariates. Arrows indicate significant effect modification ($p<0.05$).

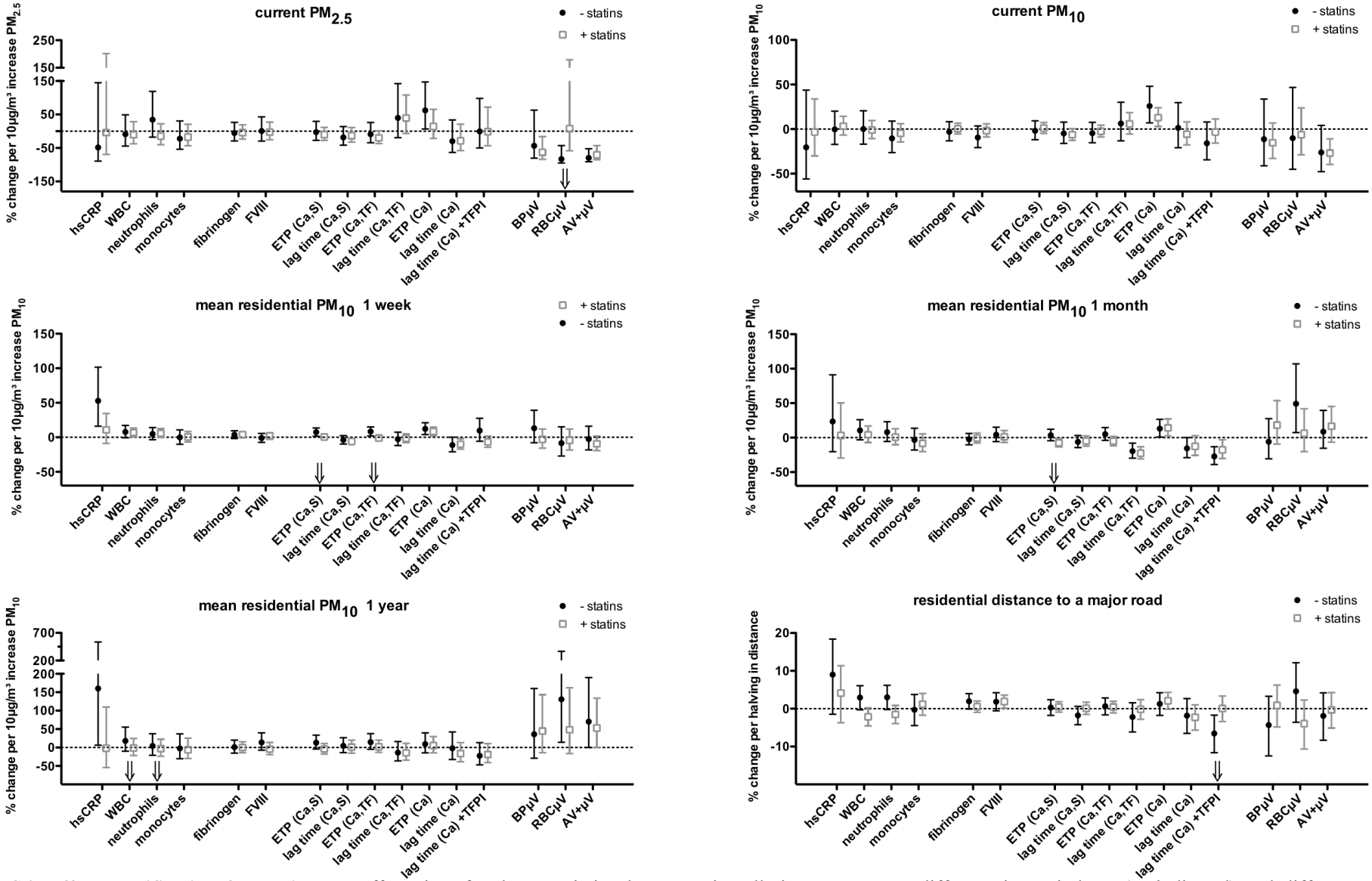

Figure S6: Effect modification for statin use. Effect sizes for the association between air pollution exposure at different time windows (as indicated) and different parameters were stratified for patients not taking statins (black) vs patients taking statins (grey). Analysis adjusted for covariates. Arrows indicate significant effect modification ($p<0.05$).

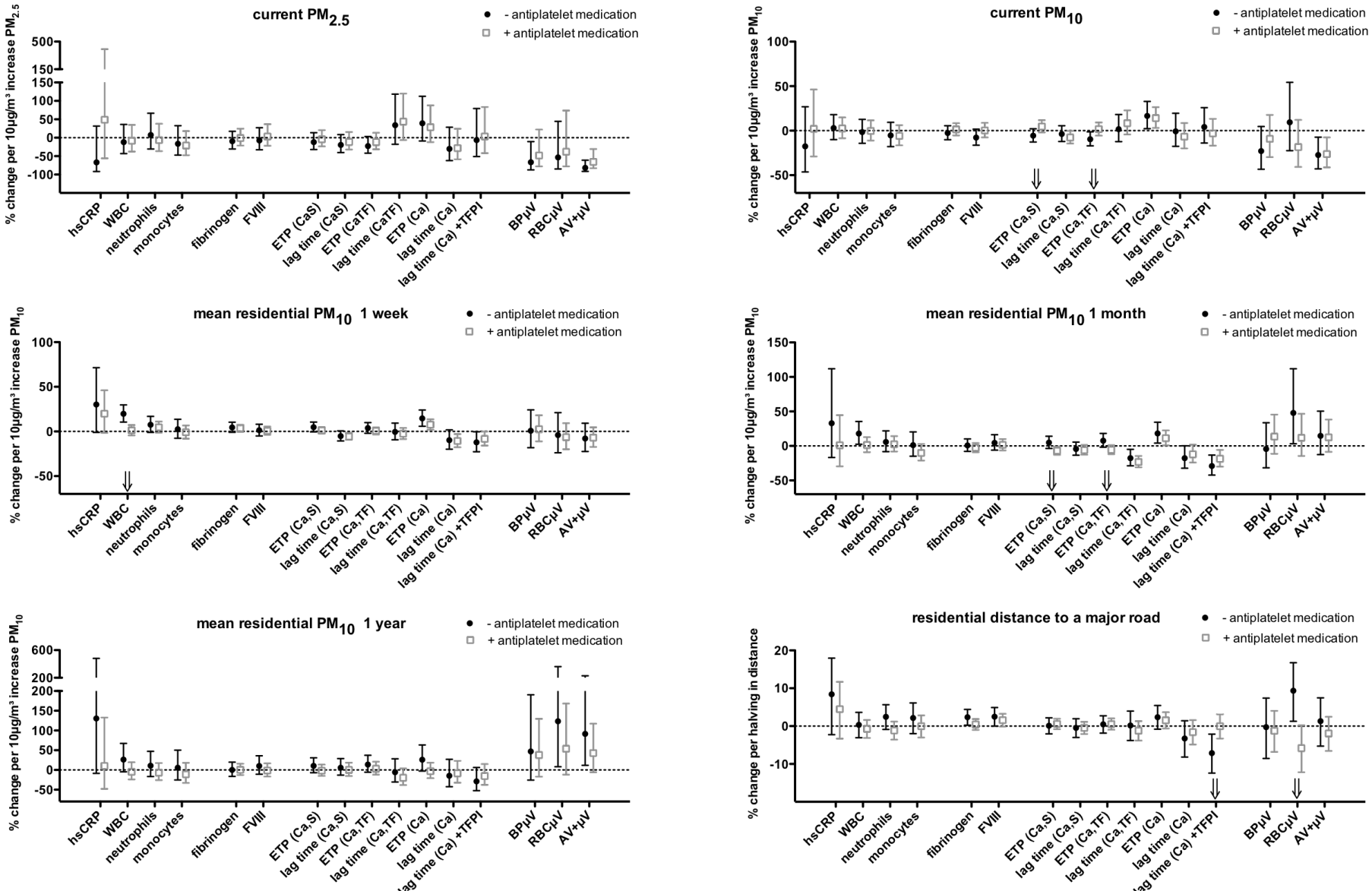

Figure S7: Effect modification for use of antiplatelet medication. Effect sizes for the association between air pollution exposure at different time windows (as indicated) and different parameters were stratified for patients not taking antiplatelet medication (black) vs patients taking antiplatelet medication (grey). Analysis adjusted for covariates. Arrows indicate significant effect modification ($p<0.05$).

CHAPTER 3

Microparticle Number or procoagulant Activity are not upregulated in healthy elderly Persons

J. Emmerechts, S. Loyen, M.F. Hoylaerts

Adapted from

Thromb Res 2011; DOI:10.1016/j.thomres.2011.09.006, epub ahead of print

Abstract

Background: Aging has been associated with an increase in the risk of venous thrombo-embolism. Elderly persons demonstrate higher plasma concentrations of different coagulation factors, but whether aging is associated with changes in the levels of circulating procoagulant microparticles (MP) remains to be investigated.

Materials and methods: We analyzed blood cell counts, coagulation and thrombin generation parameters in a cohort of young persons (n=18), and compared the results with those from a cohort of presumably healthy elderly persons (n=18), recruited from 2 nursing homes. In addition, the number and the procoagulant potential of MP was investigated by by flow cytometry and thrombin generation assays.

Results: Red blood cell counts and hemoglobin levels are lower in elderly persons, but no differences were observed in the number of total white blood cells. Higher levels of FVIII (about 60% increase in elderly vs young) and fibrinogen (about 50% increase in elderly vs young) were measured in the elderly, resulting in a shortening of the aPTT and an increase in peak height in thrombin generation assays. Plasma antigen levels of VWF almost doubled in the elderly as compared to the young.

No age-related differences were measured in the number of red blood cell-derived or blood platelet-derived MP, nor in assays that are sensitive to the surface expression of tissue factor or negatively charged phospholipids on MP.

Conclusion: Our findings, based on a cohort of healthy elderly persons outside a hospital setting, confirm that aging is associated with increases in coagulation factors, but does not elevate MP numbers or their procoagulant potential.

Introduction

Aging has been associated with an increase in the risk of venous thrombo-embolic events, including deep vein thrombosis and pulmonary embolism. Advanced age leads to a state of hypercoagulability in the blood, raising the relative risk for deep vein thrombosis (DVT) from about 1/10,000 at the age of 40 to 1/100 at the age of 80 (1). Aging may be associated with an increased prevalence of conventional risk factors, development of new, age-specific risk factors and accumulation of risk factors with age (2). Immobility, malignancy, co-morbidity, hormone replacement therapy and endothelial dysfunction all contribute to the prothrombotic risk in the elderly, in conjunction with a hypercoagulable state in the plasma (2). Elevation of the plasma levels of fibrinogen, homocystein, D-dimers, coagulation factors VII (FVII), FVIII and FIX and of plasminogen activator inhibitor 1 (PAI-1), an inhibitor of fibrinolysis, have been described (2-5). Since these procoagulant changes are not accompanied by a proportional parallel rise in natural anticoagulant factors, the haemostatic balance between pro- and anticoagulant pathways is shifted towards an elevated risk for DVT in the elderly (2).

Microparticles (MP) are circulating vesicles with a mean diameter smaller than 1 μm that are released from stimulated or apoptotic cells in the vascular bed. They carry a procoagulant potential via 2 mechanisms: the expression of negatively charged phospholipids and/or tissue factor (TF) on their membranes creates a procoagulant surface on which coagulation factors can bind and be activated to promote coagulation (6). Elevated numbers of circulating MP have been demonstrated in patients with VTE (7). We aimed to investigate whether MP numbers or their procoagulant potential increase with age.

Materials and methods

Study population

The study was designed as a pilot study to analyze the influence of age on circulating MP numbers and their procoagulant potential and was approved by the Ethics Review Board of the Medical Faculty of the University of Leuven (K.U.Leuven). We compared a cohort of young persons with a cohort of presumably healthy elderly persons. Participants gave informed consent at recruitment. To overcome possible

confounding by other risk factors such as immobilization and co-morbidity (2), elderly persons were not recruited from the hospital but from 2 nursing homes in the region of our laboratory. They were invited to participate, in writing. On the study day, a questionnaire was completed through a personal interview to collect information on general health and well-being. Medication use was registered via the nursing homes' medication files for the elderly, and by questionaire for the young persons. Exclusion criteria were immobilization, current smoking (last 6 months), use of anticoagulant therapy, diabetes, history of malignancy and self-reported non-well being at the time of blood collection (negative answer to the question 'do you feel in good health at this moment?'). Of the 26 elderly persons who agreed to participate, 4 were excluded because of use of anticoagulant medication, 2 because of diabetes, 1 because of non-well being at the time of blood collection and 1 because of difficulties during blood sampling. Young persons were recruited in the laboratory on the same days and fulfilled the same exclusion criteria.

Blood sampling and blood cell count

Non-fasting blood samples were collected using a 21 gauge needle (Terumo, Leuven, Belgium) on EDTA or on sodium citrate (3.8%) tubes (BD Vacutainer, BD Biosciences, Erembodegem, Belgium). Blood cell counts and differential leukocyte counts were determined using an automated cell counter with flow differential (Cell Dyn 3500, Abbott Diagnostics, Abbott Park, IL, USA). Citrated samples were centrifuged for 10 minutes at 1900 x g, followed by a second centrifugation step of 20 minutes at 1900 x g, within one hour after collection.

Coagulation

Activated partial thromboplastin time (aPTT), prothrombin time (PT), FVII, FVIII, fibrinogen, antithrombin (AT) and protein C (PC) were all measured via functional assays on a BCS-XP coagulation analyzer according to the manufacturer's procedures, using the manufacturer's reagents (Siemens, Hamburg, Germany).

Thrombin generation

Thrombin generation (TG) was measured by means of the Calibrated Automated Thrombography method using a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland). Thrombinoscope software (Thrombinoscope BV, Maastricht, The

Netherlands) was used to calculate thrombin generation curves, from which the lag time, the endogenous thrombin potential (ETP) and the peak height were derived. As explained in more detail in the online data supplement, TG was measured upon simple recalcification of plasma in the presence of an excess (4 μM) of exogenous phospholipids to investigate the effect of MP-contained factors on TG initiation. In addition, lag times were measured upon the addition of 300 ng/mL (final concentration) tissue factor pathway inhibitor (TFPI, R&D Systems, Abingdon, UK) to determine the contribution of tissue factor to thrombin generation. In TG assays, lag times are to a large extent determined by the amount of tissue factor (TF) present in the assay (8). Hence, measuring lag times in the presence and absence of TFPI enabled us to specifically investigate plasma tissue factor activity (see online data supplement).

Antigen measurements

ELISA was performed to measure antigen levels of TF (American Diagnostica, Stamford, USA) and von Willebrand factor (VWF, in-house). All coagulation assays were performed on thawed (-80°C) plasma.

Microparticle analysis

MP were analyzed by flow cytometry following a protocol, standardized by the Scientific and Standardization Subcommittee (SSC) of the International Society of Thrombosis and Haemostasis (ISTH) with some adaptations (9). To 20 μL of plasma, fluorescein isothiocyanate-labeled mouse anti-CD42a (BD Biosciences, Erembodegem, Belgium), phycoerythrin-labeled mouse anti-glycophorein A (GPA, BD Biosciences, Erembodegem, Belgium) and allophycocyanin-labeled annexin V (AV, Immunotools, Friesoythe, Germany) were added and samples were analyzed on a FACSCantoII flow cytometer (BD Biosciences, Erembodegem, Belgium) to define blood platelet-derived MP (PMP), red blood cell-derived MP (RBC-MP), and the procoagulant potential of these particles by measuring annexin V (AV) binding to the negatively charged phospholipids expressed on their surface. Samples were analyzed for 3 minutes at a low flow rate (0.5 μL/sec).

To also functionally assess the surface expression of negatively charged phospholipids on microvesicles, additional TG assays were performed: peak height

was measured both before and after filtering the plasma through a 0.1 μm filter (Millex-VV 0.1 μm, Millipore, Brussels, Belgium), in the presence of 5 pM exogenous TF (Innovin, Siemens, Marburg, Germany) and of 2 IU/mL recombinant FVIII (Kogenate, Bayer, Diegem, Belgium), in the absence of exogenous phosholipids. Under these conditions, TG is independent of changes in plasma FVIII levels and becomes dependent on the concentration of negatively charged phospholipids present in the plasma (see online data supplement). Therefore, the difference in peak height before and after filtering plasma samples is a specific measure of the expression of procoagulant phospholipids on the MP's membranes ('phospholipid-dependent TG'). Since freezing-thawing potentially affects the surface expression of negatively charged phospholipids on MP (10-12), all MP analyses were performed on fresh plasma.

Statistics

Statistical significance (defined as $p<0.05$) between groups was analyzed via Mann Whitney testing, using GraphPad Prism version 4.0b and Instat version 3 (GraphPad Software, San Diego, US).

Results

Table 1 compares the population characteristics and the results for blood cell count, coagulation parameters and for microvesicle analysis between young persons and elderly persons. Red blood cell (RBC) counts and hemoglobin (Hgb) levels are lower in elderly persons, but no differences were observed in the number of total white blood cells or neutrophils. In agreement with previous observations (3, 13-15), higher levels of FVIII (about 60% increase in elderly vs young) and fibrinogen (about 50% increase in elderly vs young) were measured in the elderly, resulting in a shortening of the aPTT and an increase in peak height in thrombin generation assays. Plasma antigen levels of VWF almost doubled in the elderly as compared to the young.

Both in the young and in the elderly, lag times prolonged significantly upon addition of TFPI in the assay. This observation justifies the use of lag times in TG assays as a functional measure of TF activity in the plasma (8). Despite a significant increase in the antigen levels of TF (about 40%) in the elderly as compared to the young, no age-

related shortening of the lag time was observed in TG assays, in the presence or absence of TFPI. These findings exclude upregulation of TF activity in plasma with aging. In plasma, MP are a major source of blood-borne TF activity.

MP not only exert their procoagulant activity through surface TF expression, but also through the exposure of negatively charged phospholipids on their membranes. We assessed the surface expression of procoagulant phospholipids both via flow cytometric analysis of annexin V binding, and via a modified phospholipid-dependent TG assay where differential peak height was measured in plasma before and after removal of MP through filtration. Both assays (total annexin V positivity in flow cytometry vs. differential peak height in TG) correlated strongly (r^2 0.57, $p<0.0001$) (see online data supplement). No significant changes were observed between the elderly and the young in the total number of PMP or RBC-MP, nor in their respective annexin V-binding fractions. In other words, phosholipid-dependent TG was not enhanced in the elderly.

Discussion

Our findings are in relative agreement with two recent reports on the association between MP and aging. Forest et al. demonstrated lower numbers of endothelial cell-derived MP, but no changes in the numbers of PMP or RBC-MP or in the annexin V-binding capacity of MP in the elderly in stable conditions. Annexin V-binding decreased in the elderly in septic conditions (16).

We did not assess endothelial MP in the current study because of the low number in healthy individuals. An association between phospholipid-dependent TG and age was demonstrated by Owen et al. in patients with a history of venous thromboembolim (VTE), but not in control persons without a history of VTE. However, no association was found between annexin V-binding PMP and aging for either category of persons (17). Both latter studies were performed on frozen plasma, obtained from either persons who where either hospital inpatients (16) or outpatients (17). Our study is novel because a cohort of healthy elderly persons was recruited outside a hospital setting, reducing the possibility of confounding by co-morbidity. Moreover, in order to minimize potential bias that can be introduced by freezing and thawing MP (10-

12), we chose to assay only fresh plasma to assess phospholipid-dependent procoagulant activity of MP.

In brief, our findings, based on a cohort of healthy elderly persons outside a hospital setting, confirm that aging is associated with increases in coagulation factors, but does not elevate MP numbers or their procoagulant potential.

Funding

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Conflic of interest statement

The authors declare no conflict of interest

Acknowledgement

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Table 1	YOUNG		ELDERLY		
	Median	(P25-P75) or (%)	Median	(P25-P75) or (%)	p-value
Population characteristics					
n	18		18		
Age (years)	29	(26.0-32.0)	85	(83.5-88.0)	
Gender (male)	10	(56%)	7	(39%)	
BMI (kg/m^2)	22.6	(19.7-25.4)	25.2	(23.3-27.5)	
Medication:					
Statins	0	(0%)	3	(17%)	
Antiplatelet	0	(0%)	7	(39%)	
Antihypertensive	0	(0%)	11	(61%)	
Blood cell count					
WBC ($10^3/\mu l$)	6	(4.8-7.1)	6.3	(5.6-7.1)	ns
neutrophils ($10^3/\mu l$)	3	(2.1-4.2)	3.9	3.4-4.7	ns
RBC ($10^6/\mu l$)	5.1	(4.6-5.2)	4.4	(4.2-4.9)	0.0188
HGB (g/dl)	15.3	(14.0-15.5)	13.8	(12.9-14.7)	0.0096
BP ($10^3/\mu l$)	261	(219-300)	258	(199-337)	ns
Coagulation					
aPTT (sec)	28.1	(26.6-31.4)	26.1	(25.1-27.9)	0.0397
PT (sec)	8.4	(7.8-8.7)	8.4	(7.8-8.8)	ns
Fbg (g/L)	2.2	(1.83-2.98)	3.4	(2.40-3.50)	0.0174
FVII (%)	133	(117-178)	124	(111-140)	ns
FVIII (%)	90	(77-101)	151	(138-169)	<0.0001
AT (%)	115	(108-121)	115	(99-119)	ns
PC (%)	123	(116-141)	129	(109-133)	ns
TF ag (pg/mL)	189	(147-287)	263	(220-305)	0.0398
VWF ag (ng/mL)	6097	(5006-8316)	11898	(9558-14771)	<0.0001
TG lag time (min.)	26.2	(21.8-42.0)	22.8	(18.1-60.0)	ns
TG ETP (nM)	829	(670-1417)	1299	(1078-1504)	ns
TG peak (nM)	129	(108-213)	249	(188-317)	0.0148
TG lag time +TFPI (min.)	60	(56.2-60.0)	57.5	(40.1-60.0)	ns
Microparticles					
BP derived (CD42+)					
total (count)	3488	(2309-6142)	3619	(2128-4462)	ns
AV- (count)	3178	(2133-5546)	3328	(1945-4231)	ns
AV+ (count)	184	(157-282)	255	(156-339)	ns
RBC derived (GPA+)					
total (count)	281	(194-1507)	305	(243-600)	ns
AV- (count)	75	(41-119)	117	(73-165)	ns
AV+ (count)	174	(108-268)	198	(139-360)	ns
phospholipid-dependent TG	26.6	(16.1-46.2)	22.8	(12.7-36.3)	ns

P25: 25% percentile, P75: 75% percentile, BMI: body mass index, WBC: white blood cells, RBC: red blood cells, Hgb: hemoglobin, BP: blood platelets, aPTT: activated partial thromboplastin time, PT: prothrombin time, Fbg: fibrinogen, F: factor, AT: antithrombin, PC: protein C, TFag: tissue factor antigen, VWFag: von Willebrand factor antigen, TG: thrombin generation, ETP: endogenous thrombin potential, TFPI: tissue factor pathway inhibitor, AV: annexin V, GPA: glycophorin A, ns: not significant. Statistics were perfomend using Mann Whitney analysis.

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Data supplement

Supplemental methods and results

Thrombin generation assays

Thrombin generation (TG) was measured by means of the Calibrated Automated Thrombography method using a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland). Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) was used to calculate thrombin generation curves, from which different parameters were derived:

Lag time: the time in minutes from the start of the assay to the intial generation of thrombin (the moment at which 10 nM thrombin is formed).

Endogenous thrombin potential (ETP): area under the thrombin generation curve (AUC), expressed in nM x min.

Peak height: Value for the maximum thrombin generation, expressed in nM.

Thrombin generation (TG) assays were performed in 2 different ways.

1) To measure endogenous triggers of coagulation.

TG was measured upon simple recalcification of thawed plasma in the presence of an excess (4 μM) of exogenous phospholipids. Under these conditions, TG is initiated by triggers contained in the plasma, such as contact activators and/or functional tissue factor. Lag time, endogenous thrombin potential (ETP) and peak height were derived from the thrombin generation curves. Coefficients of variation for within run and between run precision were smaller than 11% for all 3 parameters.

To evaluate the contribution of TF to TG more specifically, additional TG measurements were performed, including the addition of 300 ng/mL (final concentration) tissue factor pathway inhibitor (TFPI, R&D Systems, Abingdon, UK). Hence, while lag times, measured in the absence of TFPI, are to a large extent determined by the amount of tissue factor (TF) present in the assay (1), lag times, measured in the presence of TFPI are independent of plasma TF concentrations. Fig. S1 demonstrates how lag times shorten with increasing doses of TF and prolong upon addition of TFPI.

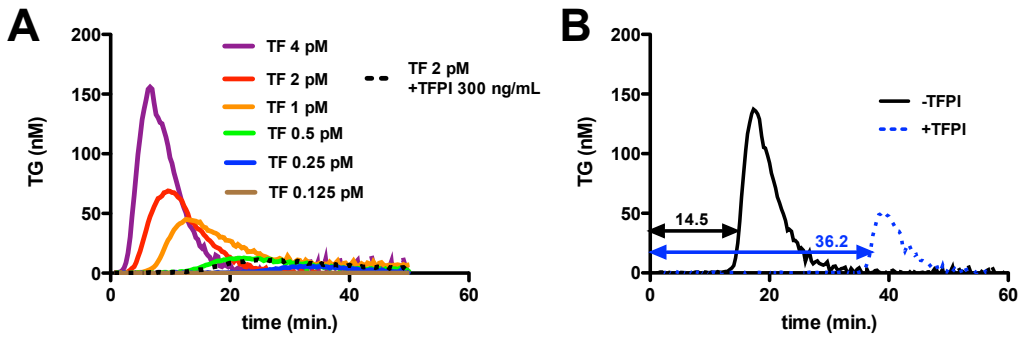

Figure S1: correlation between lag times in thrombin generation assays and tissue factor concentrations and inhibition by TFPI.
(A) Blood was collected on sodium citrate (3.8%) and corn trypsin inhibitor (CTI, 10 μg/mL blood) to block contact activation. Thrombin generation (TG) was measured in plasma at increasing concentrations (as indicated) of exogenously added tissue factor (TF, Innovin, Siemens, Marburg, Germany) in the presence of 4 μM of exogenous phospholipids (colored TG curves). A dose-dependent shortening of the lag times is observed.
The addition of tissue factor pathway inhibitor (TFPI, 300 ng/mL) is shown here in combination with 2 pM TF (black dotted line): TG is almost completely inhibited. At lower concentrations of TF, TG was completely inhibited by TFPI (not shown).
(B) TG was measured upon simple recalcification of the plasma, in the presence of 4 μM of exogenous phospholipids and in the presence (blue) or absence (black) of TFPI (representative example of the analyses performed in the present study). Plasma samples of study subjects in the present study were not collected on CTI. Correspondingly, TFPI (300 ng/mL) inhibits TG partially, with a clear prolongation of the lag time (arrows) in the filtered vs the non-filtered plasma.

2) To measure procoagulant phospholipids

Microparticles are cell-derived vesicles with a mean diameter between 0.1 and 1 μm. Through the expression of negatively charged phospholipids on their membranes, they create a procoagulant surface on which coagulation factors can bind to promote coagulation (2).

We adopted TG to functionally measure the surface expression of negatively charged phospholipids on the microparticles' surface. TG was triggered with 5 pM exogenous TF (Innovin, Siemens, Marburg, Germany) in the absence of exogenous phospholipids. Under these conditions, TG is dependent on the negatively charged phospholipids present in the plasma. Plasma was assessed both before and after removal of microparticles (Fig. S2) by filtering the plasma through a 0.1 μm filter (Millex-VV 0.1 μm, Millipore, Brussels, Belgium). The difference in TG peak height (delta peak height) between the filtered and the non-filtered plasma was used as a measure of microparticle-contained negatively charged phospholipids (called 'phospholipid-dependent TG').

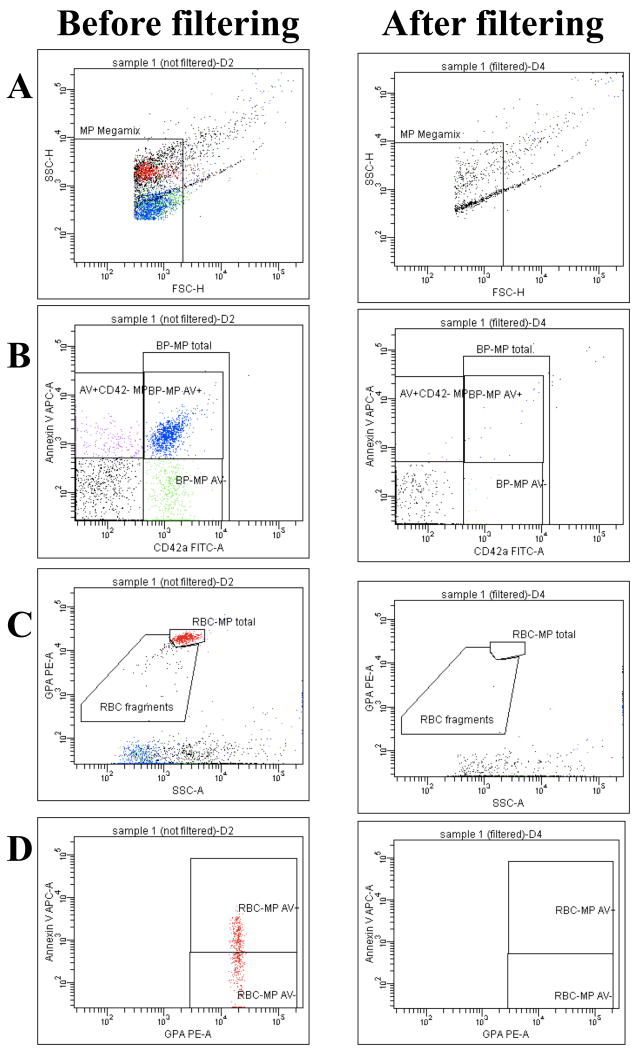

Figure S2. Removal of microparticles by filtering, as evidenced by flow cytometry.
Plasma was collected and handled as described in the main article. Both blood platelet-derived microparticles (BP-MP) and red blood cell-derived microparticles (RBC-MP) can be detected in the plasma before filtering (left panel). All MP populations disappear upon filtering of the plasma through a 0.1 μm filter (right panel). A: forward scatter (FSC-H) - side scatter (SSC-H) plot, indicating the MP gate defined by fluorescent calibrated sub-micrometer beads (Megamix, BioCytex, Marseille, France), as proposed by the standardization protocol of the International Society on Thrombosis and Haemostasis (3). B: plot showing positivity for the FITC-labelled BP marker anti-CD42a vs APC-labelled annexin V (AV). C: plot showing positivity for the PE-labelled marker glycophorin A (GPA) vs side scatter (SSC-A). A separate gate defines the position of RBC fragments. D: plot showing positivity for the PE-labelled marker glycophorin A (GPA) vs APC-labelled annexin V (AV). Only events appearing in the 'RBC-MP total' gate in plot C are shown in plot D.
Analysis performed on thawed plasma.

Since the filtering procedure also partially removes FVIII from the plasma samples (by about 50%, without altering other coagulation factor levels, data not shown), in conjunction with partial removal of von Willebrand factor, 2 IU/mL of recombinant human FVIII (Kogenate, Bayer, Diegem, Belgium) was added to both in the filtered and in the non-filtered sample, eliminating FVIII dependence (Fig. S3).

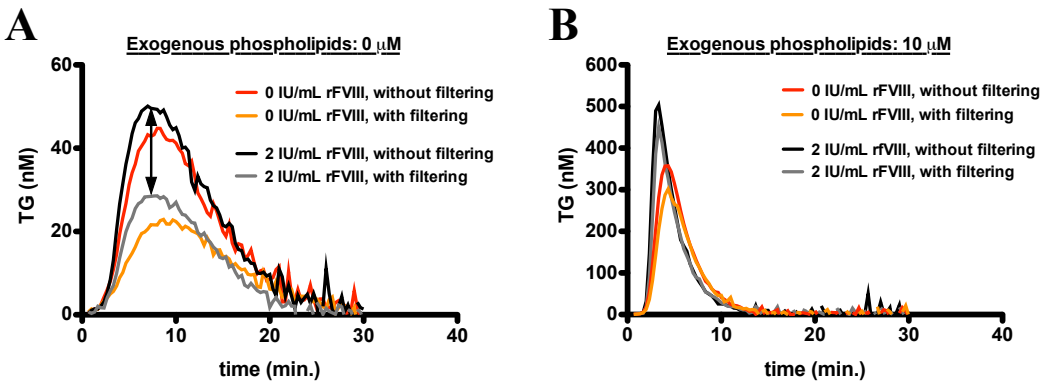

Figure S3: effect of filtering and addition of rFVIII on thrombin generation.
Thrombin generation (TG) in plasma was triggered with 5 pM tissue factor (Innovin, Siemens, Marburg, Germany), in the absence of exogenous phospholipids (A) or in the presence of 10 μM exogenous phospholipids (B). In both conditions, TG was assessed in plasma with or without filtering through a 0.1 μm filter to remove microparticles, and with or without the addition of recombinant human FVIII (rFVIII), as indicated.
In the absence of exogenous phospholipids (A), the differencee in peak height observed between the filtered (orange) and the non-filtered (red) plasma can be attributed to lower concentrations of both FVIII and endogenous phospholipids in the filtered sample. Therefore, an excess of rFVIII was added to both the non-filtered (black) and the filtered (grey) plasma to make the assay more phospholipid-specific.
We define the 'phospholipid-dependent TG' as the difference in peak height (delta peak) between the 2 latter conditions (arrow).
Panel (B) demonstrates how differences in peak height between the non-filtered (black) and the filtered (grey) condition disappear in the presence of an excess of both exogenous phospholipids (10 μM) and rFVIII (2 IU/mL).

The surface expression of negatively charged phospholids on microparicles was also assessed by measuring binding to annexin V by flow cytometry (see main article). We found a strong correlation between the 'phospholipid-dependent TG assay' and the flow cytometric measurement of annexin V binding ($p<0.0001$, r^2 0.57) for the samples in the present study (Fig. S4).

Freezing-thawing potentially affects the surface expression of negatively charged phospholipids on MP (4-6), therefore, all MP analyses were performed on fresh plasma.

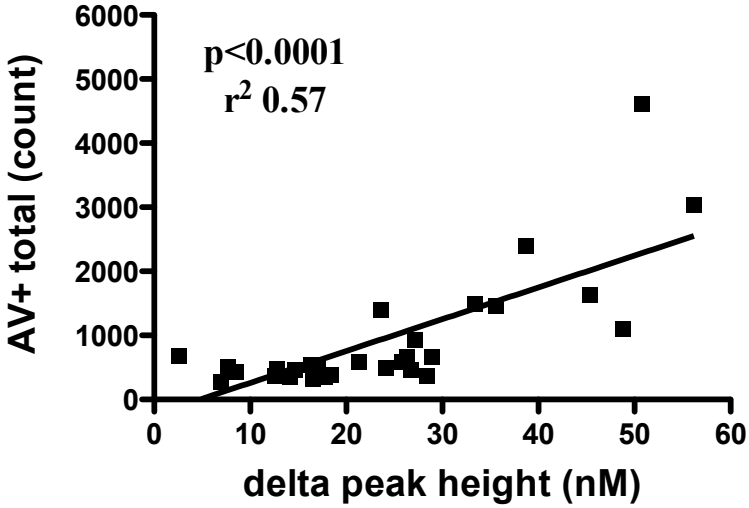

Figure S4. Correlation between annexin V binding and phospholipid-dependent thrombin generation.
Binding of annexin V (AV) to the negatively charged phospholipids on the microparticles' surface was measured by flow cytometry (Y-axis), and correlated to the results of the functional measurement in the phospholipid-dependent thrombin generation (TG) assay (based on the difference in peak height (delta peak height, X-axis) between filtered and non-filtered plasma).
Data shown are obtained from fresh plasma of all study subjects in the present study.

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CHAPTER 4

Hemostatic changes in young and old mice upon subchronic exposure to air pollution in an urban roadside tunnel

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Abstract

Objective: Elderly subjects are particularly susceptible to the cardiovascular effects of air pollution. We investigated if traffic-related air pollution exposure alters hemostatic parameters differently in young and old mice.

Methods and results: Young (10 weeks) and old (20 months) mice were placed in an urban roadside tunnel or in a clean environment for 25 or 26 days.

Tunnel mice showed an elevated carbon load and endothelial von Willebrand factor (VWF) expression in the lung, but no pulmonary or systemic inflammation. Traffic-related air pollution raised red blood cell and blood platelet numbers in young and old mice, but raised numbers of procoagulant blood platelet-derived microvesicles only in old mice.

Baseline levels of plasma soluble P-selectin (sP-sel) and VWF were higher in old mice and did not further increase with exposure. In young mice, sP-sel increased with exposure and correlated strongly with soluble E-selectin and VWF.

Changes in coagulation factors and thrombin generation were mild or absent.

Conclusion: Traffic-related air pollution does not trigger overt lung inflammation, but triggers endothelial cell and platelet activation. The high absolute numbers reached in the aged exposed mice for blood platelets, microvesicles, sP-sel and VWF indicate higher thrombogenicity in old mice, subchronically exposed to polluted air.

Introduction

Over the last two decades, a large body of epidemiological research has consistently demonstrated an association between exposure to air pollution and cardiovascular disease and mortality (1-7). Both gaseous (carbon monoxide, nitrogen oxides, sulfur dioxide, ozone) and particulate components (particulate matter, PM) are implicated in air pollution, but the larger body of evidence points towards the deleterious effects of the particulates. Both acute and chronic cardiovascular health effects are described. Thus, exposure to PM for as little as 2 h suffices to trigger myocardial infarction (8). More chronic exposure, such as living near a major road, has been associated with increased coronary artery calcification, a measure for atherosclerosis (9), and with an enhanced risk for venous thrombosis (10). In view of the commonness of exposure in modern society, traffic-related air pollution likely poses a major public health burden (11).

Because of a gradual decline in physiological processes and a higher prevalence of pre-existing cardiovascular and respiratory diseases, elderly persons (>65 years of age) are believed to be more susceptible to deleterious cardiovascular effects of air pollution than young persons (12). PM exposure-related relative risk estimates appear to be higher in elderly, both for mortality (1, 5, 13) and hospitalization for cardiovascular disease (14, 15). Similarly, the relative risk for cardiovascular disease associated with gaseous pollutants is increased in the very elderly (≥ 75 years of age) (16). Even though an association between age and increased relative risk for air pollution-related cardiovascular disease was not found in all studies, the attributable risk, an estimate depending both on the strength of the association between risk factor and disease and its prevalence, was highest among the elderly, due to their greater baseline risk (16). Indeed, the elderly not only have a higher baseline risk for developing myocardial infarction (17), but they also show a strongly upregulated tendency for venous thrombogenicity, linked to elevation of several coagulation factors (18). Yet, despite growing epidemiological evidence of raised cardiovascular risk associations with air pollution exposure in the elderly, it is not known if air pollution affects blood hemostasis parameters differently with age.

In the current study, we investigated the effects of exposure to traffic-related air pollution on hemostasis in young vs old mice. In mice, a short-term experimental exposure via intratracheal instillation of PM was found to be inadequate to induce changes in secondary hemostasis parameters (19). Therefore, presently, we placed young and old mice in an urban roadside tunnel for 25 or 26 days, and analyzed hemostasis thoroughly, in comparison to partial and non-exposed groups. In addition to a detailed analysis of parameters of primary hemostasis, plasma was analyzed for microvesicles (also called microparticles, a term we prefer to avoid in the context of pollution by particles), since negatively charged phospholipids and tissue factor (TF) on their membranes manifest procoagulant properties, via binding of coagulation factors (20).

Materials and methods

Mice

This study was approved by the Institutional Review Board of the University of Leuven, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Male C57Bl6/j mice aged 10 weeks ('young') or 20 months ('old') were purchased from Janvier (Le Genest St Isle, France).

Experimental design

Mice were randomly allocated to 1 of 3 groups: a first group ('clean') remained in the animal facility of the University of Leuven during the whole period of the experiment. They were kept in micro-isolation cages covered by a conventional filter top (Tecniplast, Buggiate, Italy) in a light-controlled (14/10-h day/night cycle) room with continuous air filtration. The second ('tunnel filtered') and third ('tunnel exposed') groups were stationed in an urban roadside tunnel (Craeybeckx tunnel, Antwerp, Belgium) at 57 km from the University of Leuven. This tunnel is a major motorway of car and truck traffic entering Antwerp. It is 1600 m long and consists of separate pipes for each direction, each comprising 4 lanes. Mice were placed in an alcove inside the tunnel, close to the end of one of the pipes and at 4 m distance of the bypassing traffic. Preliminary measurements demonstrated that PM concentrations were highest at this location of the tunnel (data not shown). Mean daily counts for

vehicle traffic in 2010 for this pipe were 49,780 non-trucks and 6,545 trucks (source: Department Mobility and Public Works - Division Traffic Centre of the Flemish government, unpublished data). Mice allocated to the 'tunnel filtered' group were placed in micro-isolation cages covered with two layers of highly efficient flexible filter material (Pads F7, Camfil Farr, Zaventem, Belgium, minimum life efficiency on 0.4 μm: ≥35%), separated by about 5 cm of air. Mice allocated to the 'tunnel exposed' group were placed in the same location in micro-isolation cages without filter tops. For all 3 groups, mice had access to food and water *ad libitum* and cages were cleaned once a week (n=7-8 per group). The total study period was 25 to 26 days.

Exposure measurements

PM measurements were performed every 30 minutes during the entire exposure period, using a portable laser-operated aerosol mass analyzer (Aerocet 531, Met One Instruments Inc, Grant Pass, OR, USA) inside empty cages, placed among the mice-containing cages, in the tunnel and control sites.

PM concentrations were also assessed on a single occasion by sampling PM on a glass microfibre filter (Whatman, Kent, UK) by using a sampling pump (SP350, TSI, Shoreview, USA) at 2 L/min over 10 hours.

Data on the tunnel traffic during the study period were collected through courtesy of the Department Mobility and Public Works - Division Traffic Centre of the Flemish government in Belgium.

Blood collection

At the end of the exposure period (25-26 days), mice were anesthetized (sodium pentobarbital, 60 mg/kg, i.p.) and blood was collected from the retroorbital sinus on citrate 0.38% or on EDTA. Blood cell counts and differentials were performed on a Cell-Dyn 3500R counter (Abbott, Diegem, Belgium). Citrated samples were centrifuged twice (1,500 g x 15 min) and plasma was snap frozen in liquid nitrogen and stored at -80°C for subsequent coagulation and microvesicle assays. EDTA-anticoagulated blood was centrifuged once (15,000 g x 10min) and plasma was stored at -20°C for antigen measurements.

Bronchoalveolar lavage and lung tissue collection

Following blood sampling, the mouse abdomen and thorax were opened. The left bronchus was ligated and the trachea exposed and cannulated with a 20 Gauge cannula to lavage the right lung three times with 0.4 mL (total volume of 1.2 mL) of sterile NaCl 0.9%. The recovered bronchoalveolar lavage fluid (BALF) aliquots were pooled and placed on ice. No difference in the volume of collected fluid was observed between groups. Cells in fresh BALF were stained with Trypan Blue and counted in a Bürker hemocytometer. Cell differentials were determined by light microscopy on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Siemens, Brussels, Belgium). The remaining BALF was then centrifuged (1000 g x 10 min.) and the supernatant stored at -20°C for cytokine analysis.

Upon bronchoalveolar lavage, the left lung was filled with 0.4 mL parafomaldehyde 4% (PFA), isolated and immersed in PFA 4% for fixation.

Carbon load

Airway macrophages were visualized by light microscopy (AxioPlan 2 Imaging, Zeiss, Zaventem, Belgium) and pictures were taken with Axiovision Rel. 4.6. (Zeiss). ImageJ software (version 1.440, National Institutes of Health, USA) was used to calculate the carbon load of airway macrophages via surface analysis, as described previously (21, 22). First, the nucleus was removed from the image of each macrophage. Then carbon load was defined as the median area (μm^2) occupied by carbon, in at least 20 randomly selected macrophages per mouse.

Histology

Lung tissues of 3 randomly chosen mice per group were embedded in paraffin and 8 μm cross-sections were stained with hematoxylin and eosin (H&E) or von Willebrand factor (VWF) using rabbit anti-human VWF antibody and a biotinylated secondary goat anti-rabbit antibody (both Dako, Heverlee, Belgium). All slides were stained at the same time and for an identical reaction time. Stainings were analyzed with a Zeiss AxioPlan 2 Imaging microscope (Zeiss, Jena, Germany) and pictures were taken with Axiovision Rel. 4.6. (Zeiss). Per mouse, the intensity of the VWF staining was manually scored by 3 independent and blinded observers, via a simple graded score of 1, 2 or 3, corresponding to light, moderate or intense staining, respectively, for each section.

Coagulation assays

All coagulation assays were performed on the BCS-XP coagulation analyzer (Siemens, Hamburg, Germany) using the manufacturer's reagents and adapted procedures for murine coagulation testing (23). Coagulation factor (F) VII and FVIII activity were determined by one-stage coagulation assays, calibrated with pooled murine plasma. Fibrinogen concentrations were determined by the functional Clauss method, calibrated with Standard Human Plasma (Siemens, Hamburg, Germany).

Thrombin generation assays (TGA)

Thrombin generation was measured in diluted plasma (1/4) by means of the Calibrated Automated Thrombography (CAT) method using a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland). Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) was used to calculate thrombin generation curves, from which the lag times, endogenous thrombin potential (ETP) and peak height were derived. TGA were performed in two ways: first, in the presence of 10 μM phospholipids and in the absence of exogenously added tissue factor (TF) (condition called '+PL-TF'), and second, in the presence of both 10 μM phospholipids and 0.3 pM exogenously added TF (Innovin, Siemens, Marburg, Germany) (condition called '+PL+TF').

Flow cytometric microvesicle analysis

Plasma was thawed at 37°C, labeled with fluorescein isothiocyanate-labeled anti-CD41/61 (Emfret, Eibelstadt, Germany), phycoerythrin-labeled anti-Ter119 (BD Biosciences, Erembodegem, Belgium) and allophycocyanin-labeled annexin V (AV, Immunotools, Friesoythe, Germany) and analyzed on a FACSCantoII flow cytometer (BD Biosciences, Erembodegem, Belgium) to define blood platelet-derived microvesicles ('BP-μV') and red blood cell-derived microvesicles ('RBC-μV'). Surface expression of negatively charged phospholipids was assessed by measuring annexin V binding ('AV').

Antigen measurements

Von Willebrand factor antigen (VWFag) levels were measured by in-house ELISA on citrated plasma using rabbit anti-human VWF (Dako, Heverlee, Belgium).

Commercial ELISA's were used to determine the concentrations of soluble P-selectin (sP-sel), soluble E-selectin (sE-sel), platelet factor 4 (PF4) (all R&D Systems, Abingdon, UK) and interleukin-6 (IL-6, eBioscience, Vienna, Austria) oin EDTA plasma.

Statistical analysis

We used non-parametrical Kruskal-Wallis tests with Dunn's multiple comparison post-tests to analyze significance between the different exposure groups within each age class, and Mann-Whitney tests to analyze significance between both age classes for mice placed in the 'clean' environment. All statistical analyses were performed using GraphPad Prism version 4.0b (GraphPad Software, San Diego, US).

Results

Exposure characteristics

Figure 1 shows the measurements for PM_{10} (PM with a mean diameter smaller than 10 μm) and for $PM_{2.5}$ (PM with a mean diameter smaller than 2.5 μm) at both locations, together with the hourly vehicle transit through the tunnel.

In general, PM measurements parallelled vehicle transit data, with higher values on week days as compared to weekend days, and higher values during the morning rush hours. Mean levels of PM_{10}, averaged over the entire study period, were 59% lower inside the filtered cages in the tunnel ('tunnel filtered', 38.6 $\mu g/m^3$) and 97% lower inside the cages in the clean environment ('clean', 2.9 $\mu g/m^3$), compared with the unfiltered cages in the tunnel ('tunnel exposed', 93.8 $\mu g/m^3$). For $PM_{2.5}$, values were 32.3% lower for the 'tunnel filtered' group (16.8 $\mu g/m^3$) and 95.5% lower for the 'clean' group (1.1 $\mu g/m^3$), compared to the 'tunnel exposed' group (24.9 $\mu g/m^3$). Correspondingly, the collection filter was considerably blacker upon sampling of unfiltered tunnel air than upon sampling of filtered tunnel air, while hardly any particulate material was sampled on the filter in the clean environment (Fig. 1).

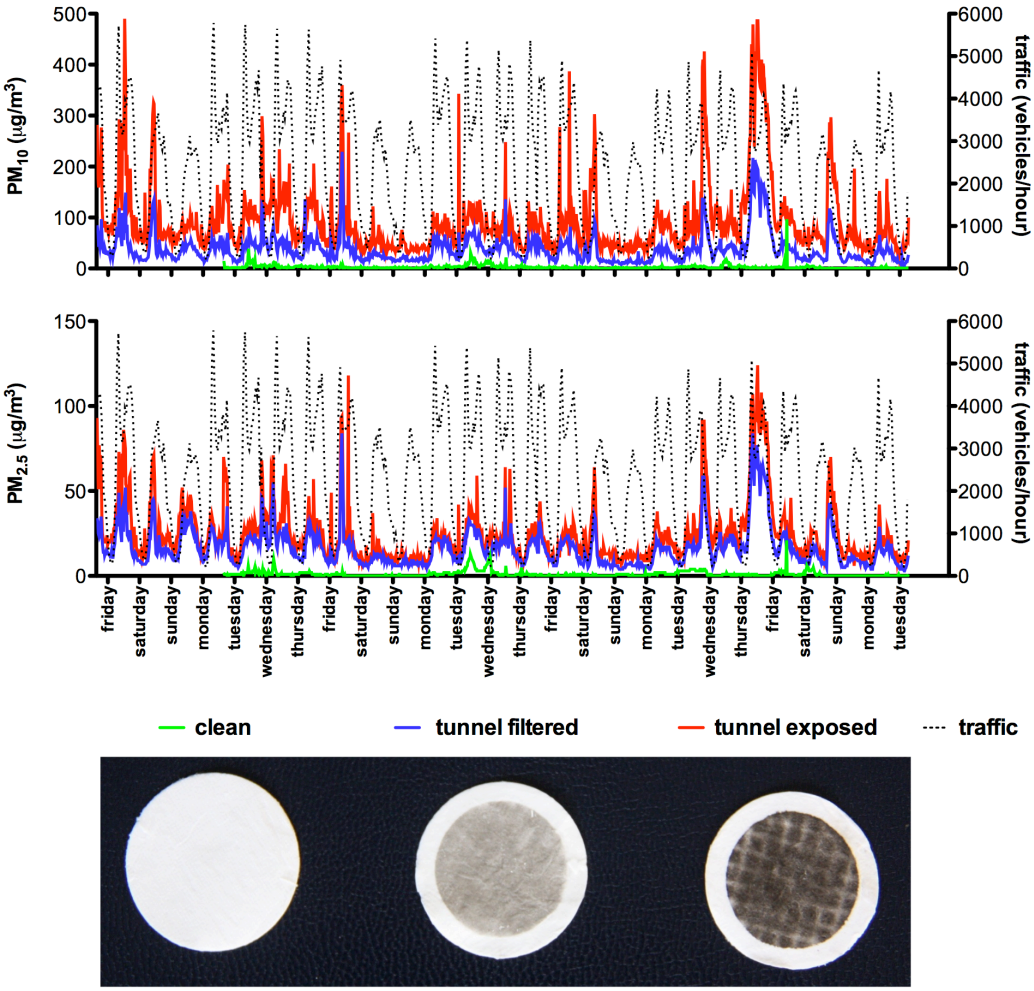

Figure 1: Exposure characteristics
Levels of PM_{10} (upper graph, left Y-axis) and $PM_{2.5}$ (lower graph, left Y-axis) were measured inside cages placed in both locations (as indicated), during the entire study period (days in X-axis). Dotted line represents total vehicle traffic transit through the roadside tunnel, calculated per hour (right Y-axis). The bottom panel shows glass microfibre filters on which PM was sampled in (from left to right) a cage in the clean environment, a filtered cage inside the roadside tunnel and an unfiltered cage inside the tunnel.

Bronchoalveolar lavage cells and IL-6

Baseline values (defined as the value for the 'clean group') of BALF WBC concentrations were higher in old mice than in young mice (Table 1). Almost all BALF cells were monocytes/macrophages. Exposure to traffic-related air pollution did not influence WBC counts or differentiation in either age category (Table 1 and Fig. 2), despite the high carbon load in alveolar macrophages of the tunnel mice, which tended to be higher for the 'tunnel exposed' than for the 'tunnel filtered' group (Fig. 2D). Concentrations of IL-6 in BALF were below the detection limit for all but 2 mice (in different groups).

Table 1	YOUNG			OLD		
	clean	**tunnel filtered**	**tunnel exposed**	**clean**	**tunnel filtered**	**tunnel exposed**
Final body weight (g)	27.8 (26.9-28.0)	28.7 (27.8-29.8)	29.0 (28.2-29.5)	**30.3 (29.7-33.8)**¶	33.4 (32.2-34.1)	31.1 (29.5-32.6)
BALF cell count						
WBC (/μL)	45.0 (25.0-50.0)	50.0 (42.5-57.5)	50.0 (32.5-60.0)	**77.5 (70.0-90.0)**¶¶	80.0 (40.0-80.0)	60.0 (42.5-85.0)
Neutrophils (%)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.8)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Monocytes (%)	100 (99.3-100.0)	99.5 (97.5-100.0)	100.0 (99.0-100.0)	99.5 (97.5-100.0)	99.0 (96.0-100.0)	98.5 (98.0-100.0)
Coagulation						
Fibrinogen (mg/dL)	199 (172-221)	188 (181-207)	196 (173-226)	191 (178-207)	181 (171-201)	192 (182-201)
FVII (%)	115 (107-125)	116 (112-137)	122 (114-127)	117 (108-127)	120 (114-140)	115 (107-129)
FVIII (%)	95 (93-112)	106 (86-119)	107 (103-125)	**134 (112-144)**¶¶	111 (103-128)	120 (111-129)
TG (+PL-TF) lagtime (min.)	9.0 (5.8-14.7)	10.7 (10.0-13.3)	14.7 (7.1-14.8)	17.3 (10.3-60.0)	13.5 (7.7-17.8)	14.3 (11.7-60.0)
TG (+PL-TF) ETP (nM.min)	102 (76-139)	123 (108-208)	105 (70-161)	67 (0-162)	61 (39-89)	143 (0-193)
TG (+PL-TF) peak (nM)	9.6 (7.0-10.5)	9.6 (7.4-18.7)	8.3 (5.0-11.5)	4.9 (0.0-11.5)	5.7 (2.9-7.9)	9.5 (0.0-20.8)
TG (+PL+TF) lagtime (min.)	2.8 (2.4-3.2)	2.9 (2.4-3.1)	3.0 (2.3-3.1)	3.5 (2.8-3.7)	3.0 (2.7-3.3)	3.5 (2.7-3.8)
TG (+PL+TF) ETP (nM.min)	296 (279-320)	**375 (350-422)****	317 (301-353)	330 (255-412)	365 (274-442)	312 (282-365)
TG (+PL+TF) peak (nM)	42.1 (37.7-48.8)	39.3 (38.1-42.1)	37.5 (35.8-40.2)	44.4 (40.1-52.9)	45.4 (35.7-48.6)	38.0 (34.7-45.6)

All data are represented as median (percentile 25 - percentile 75)
** $p<0.01$ vs 'young clean' (Kruskal Wallis), ¶ $p<0.05$ vs 'young clean', ¶¶ $p<0.01$ vs 'young clean' (Mann-Whitney)

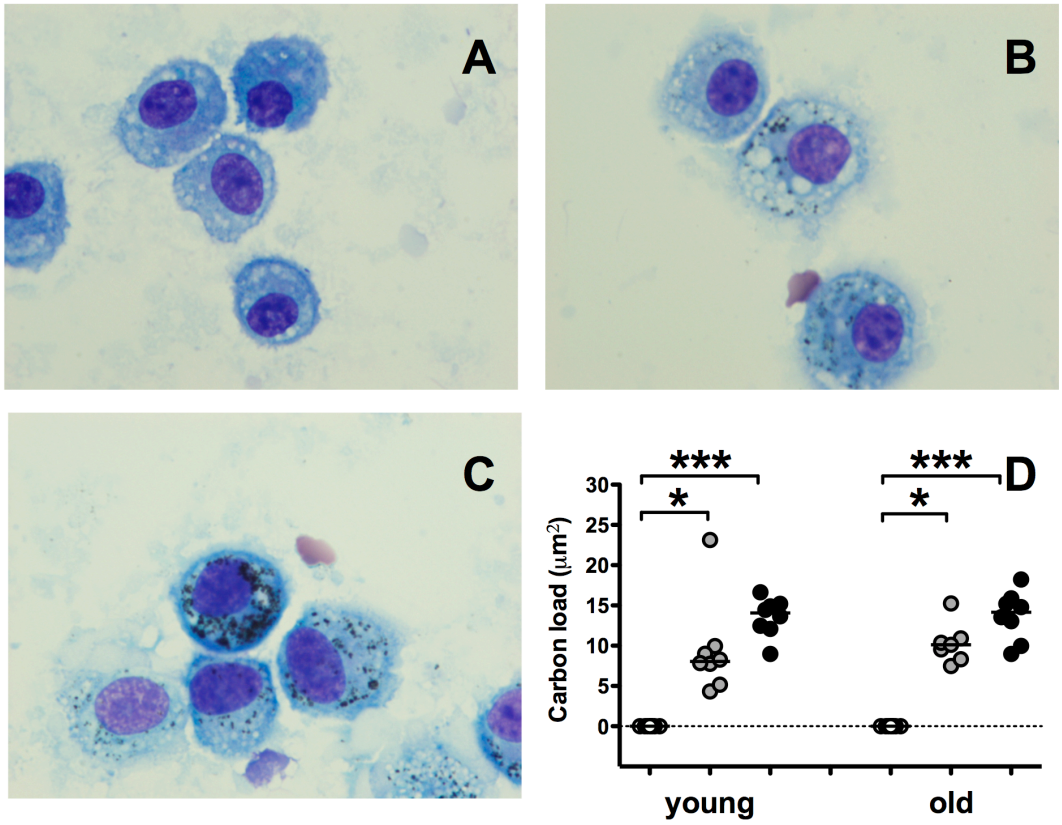

Figure 2: Microscopic images of BALF cells and carbon load
A-C: Microscopic image of cytospins of bronchoalveolar lavage fluid (BALF) cells from young mice placed in a cage in the clean environment (A), a filtered cage inside the roadside tunnel (B) and an unfiltered cage inside the tunnel (C). Carbon particles are obvious in macrophages for both tunnel groups. D: measurement of carbon load in alveolar macrophages from young (left) and old (right) mice, placed in a clean environment ('clean', white dots), in filtered cages inside the tunnel ('tunnel filtered', grey dots) or in unfiltered cages inside the tunnel ('tunnel exposed', black dots). Data are represented as a scatter dot plot with median values. Dotted line represents baseline median value (=value for the 'clean' group) for the young mice. * $p<0.05$, *** $p<0.001$.

Lung histology

No morphological differences were noted between groups on H&E stained lung sections (data not shown). Immunohistochemical staining for VWF was suggestive of higher expression, especially in the smaller pulmonary blood vessels for the 'tunnel exposed' [mean (SD) score for young mice: 2.2 (0.8), old mice: 1.8 (0.2)] and the 'tunnel filtered' groups [mean (SD) score for young mice: 2.7 (0.3), old mice: 2.4 (0.4)], as compared to the 'clean' group [mean (SD) score for young mice: 1.6 (0.7), old mice: 1.4 (0.2)] (Fig. 3).

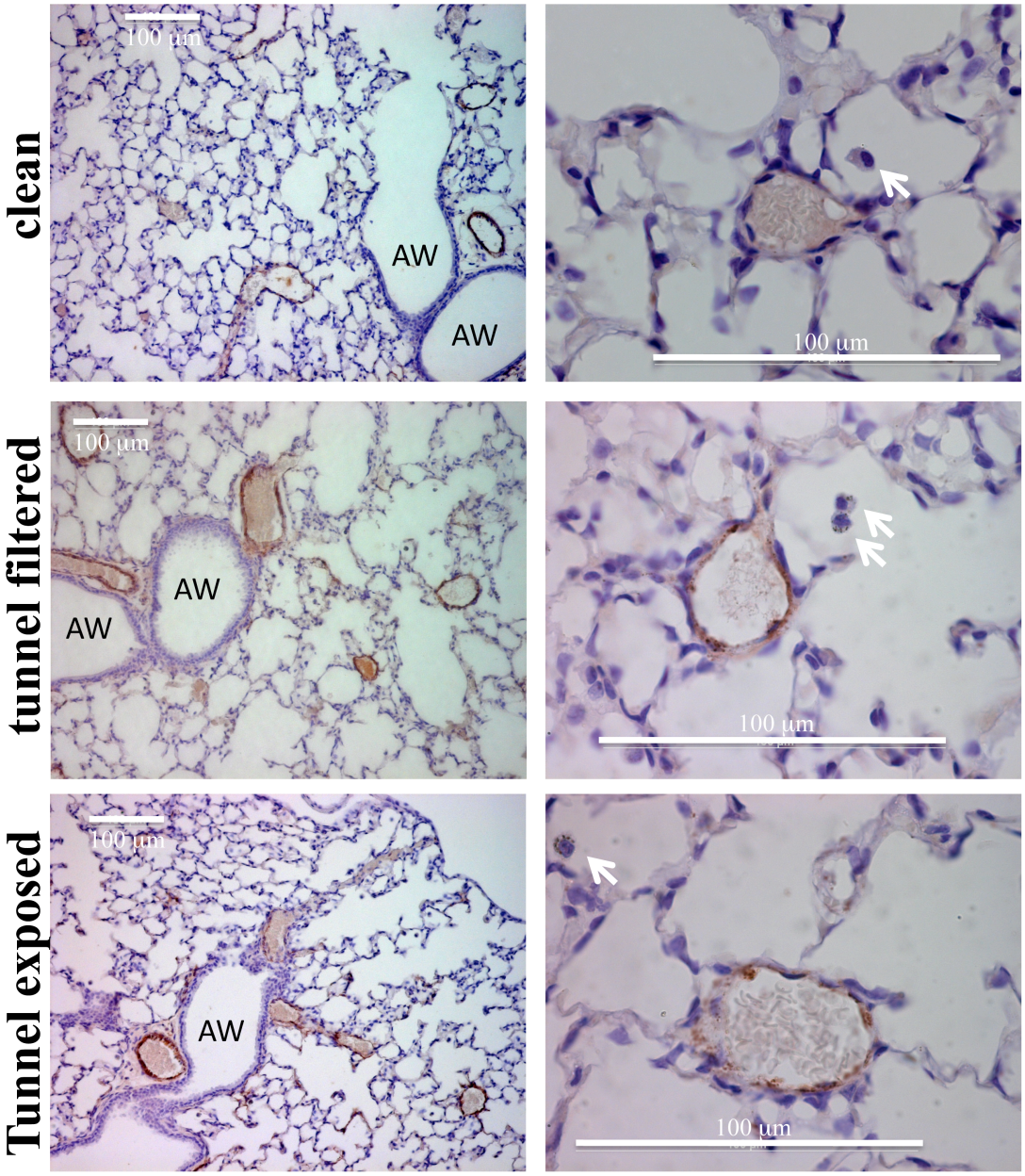

Figure 3: immunohistochemical staining for VWF in lung tissue
Representative images (left: low magnification, right: high magnification) of immunohistochemical staining for VWF in lung tissues from young mice placed in a clean environment ('clean'), in filtered cages inside the tunnel ('tunnel filtered') or in unfiltered cages inside the tunnel ('tunnel exposed'). While large blood vessels stained positive in all 3 groups, the majority of smaller blood vessels did not stain in the 'clean' group, but stained moderately to strongly positive in both tunnel groups. Arrows indicate alveolar macrophages. AW= large airways.

Hematological parameters and IL-6

Results for blood cell counts are shown in figure 4. Higher baseline values in old vs young mice were observed for WBC, RBC, hemoglobin and blood platelet levels. WBC concentrations were not affected by exposure to traffic-related air pollution. In contrast, values for RBC and hemoglobin increased significantly for the 'tunnel filtered' and 'tunnel exposed group' as compared to the 'clean' group, both in young

and old mice. BP numbers also increased in all 'tunnel' groups, with a trend to even higher values in the 'tunnel exposed' group for the old mice. Concentrations of IL-6 in plasma were below the detection limit for all but 3 mice (in different groups).

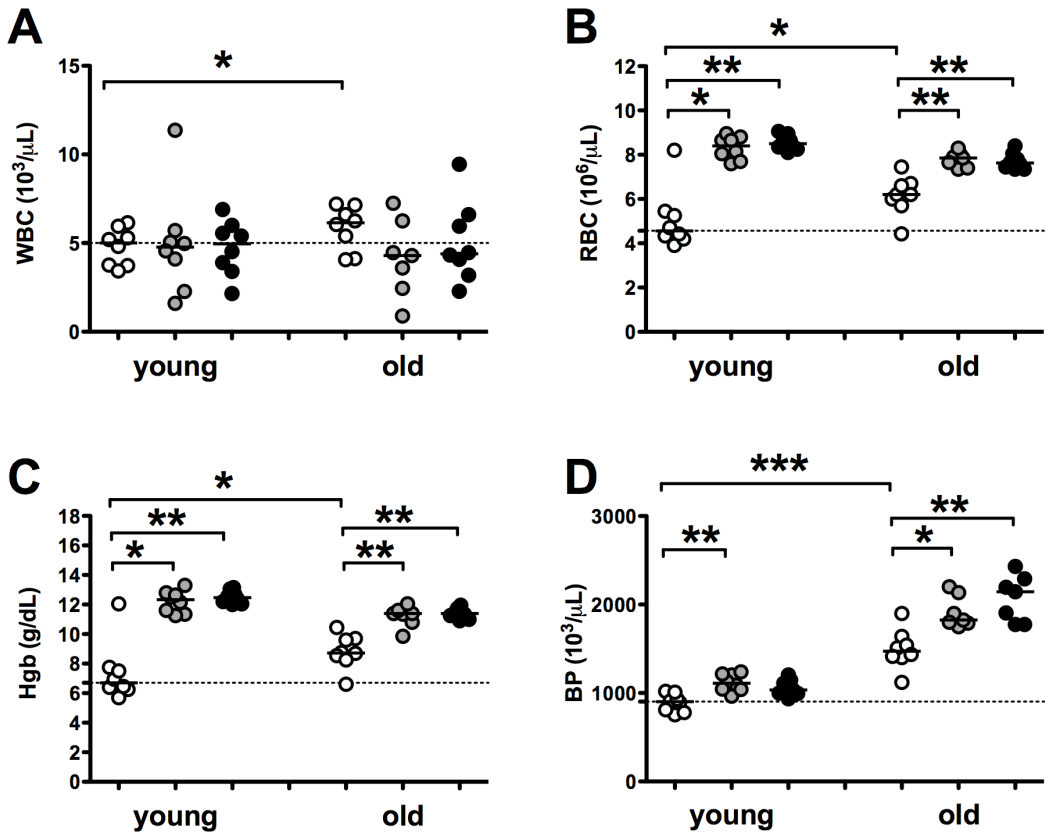

Figure 4: Blood cell parameters
(A) White blood cells (WBC), (B) red blood cells (RBC), (C) hemoglobin (Hgb) and (D) blood platelets (BP) in blood from young (left) and old (right) mice, placed in a clean environment ('clean', white dots), in filtered cages inside the tunnel ('tunnel filtered', grey dots) or in unfiltered cages inside the tunnel ('tunnel exposed', black dots). Data are represented as a scatter dot plot with median values. Dotted line represents baseline median value (=value for the 'clean' group) for the young mice.* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Markers of BP and endothelial activation

Baseline values for sP-sel and VWF were higher in old vs young mice. Exposure to traffic-related air pollution upregulated concentrations of sP-sel in young mice, with trends towards similar elevations in the concentrations of sE-sel and VWF. No differences were observed between 'tunnel filtered' and 'tunnel exposed' groups. Values in old mice were unaltered by air pollution exposure (Fig. 5). Both in young and old mice, levels of the specific endothelial marker sE-sel correlated with levels of sP-sel ($p<0.0001$, r^2: 0.605 and $p=0.0035$, r^2: 0.386 respectively) and of VWF ($p=0.02$, r^2: 0.312 and $p=0.05$, r^2: 0.200 respectively).

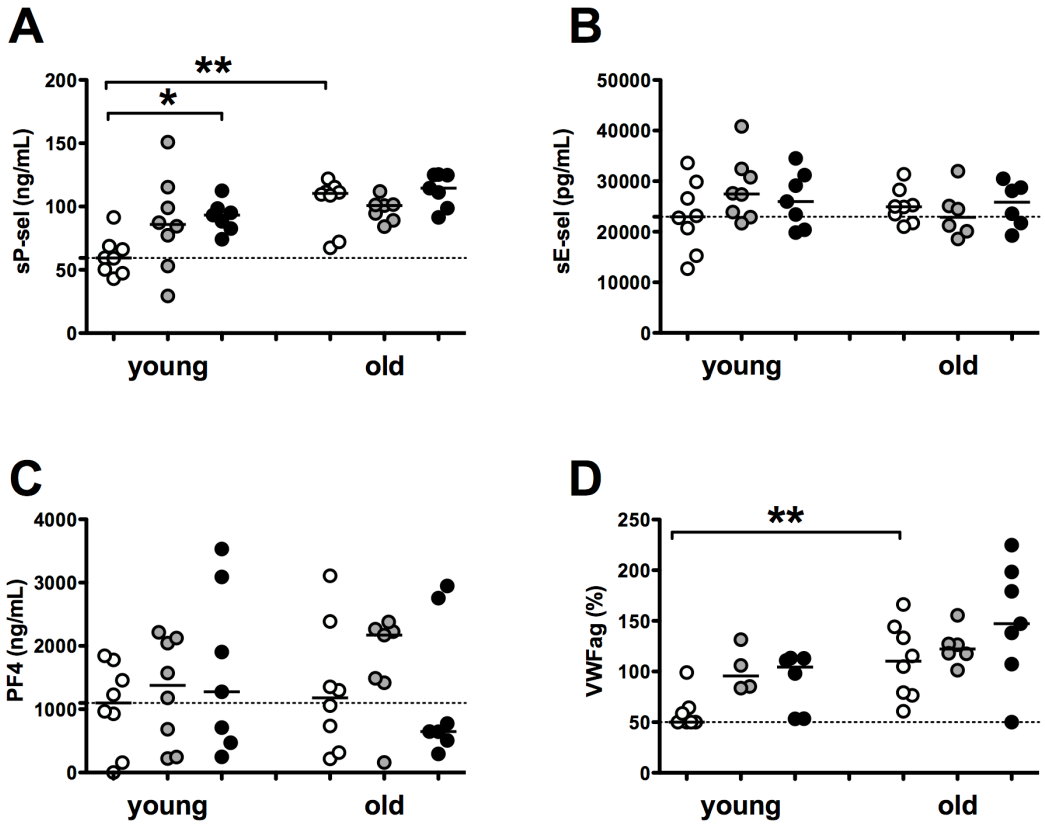

Figure 5: Markers of endothelial and blood platelet function
(A) soluble P-selectin (sP-sel), (B) soluble E-selectin (sE-sel), (C) platelet factor 4 (PF4) and (D) von Willebrand factor antigen (VWFag) levels in plasma from young (left) and old (right) mice, placed in a clean environment ('clean', white dots), in filtered cages inside the tunnel ('tunnel filtered', grey dots) or in unfiltered cages inside the tunnel ('tunnel exposed', black dots). Data are represented as a scatter dot plot with median values. Dotted line represents baseline median value (=value for the 'clean' group) for the young mice.* $p<0.05$, ** $p<0.01$.

Microvesicles

Baseline values for all investigated microvesicle populations were similar between old and young mice. The majority of microvesicles bound annexin V, a measure for their surface exposure of procoagulant negatively charged phospholipids. In contrast to the young mice, a trend towards increased levels of BP-μV (total, AV- and AV+) was observed for the old mice in the tunnel, with the highest values for the 'exposed' group (Fig. 6). Total BP-μV numbers correlated with circulating BP numbers in young mice ($p=0.01$, r^2: 0.279) and to a lesser extent in old mice ($p=0.07$, r^2: 0.171). No correlations were found between RBC-μV and circulating RBC.

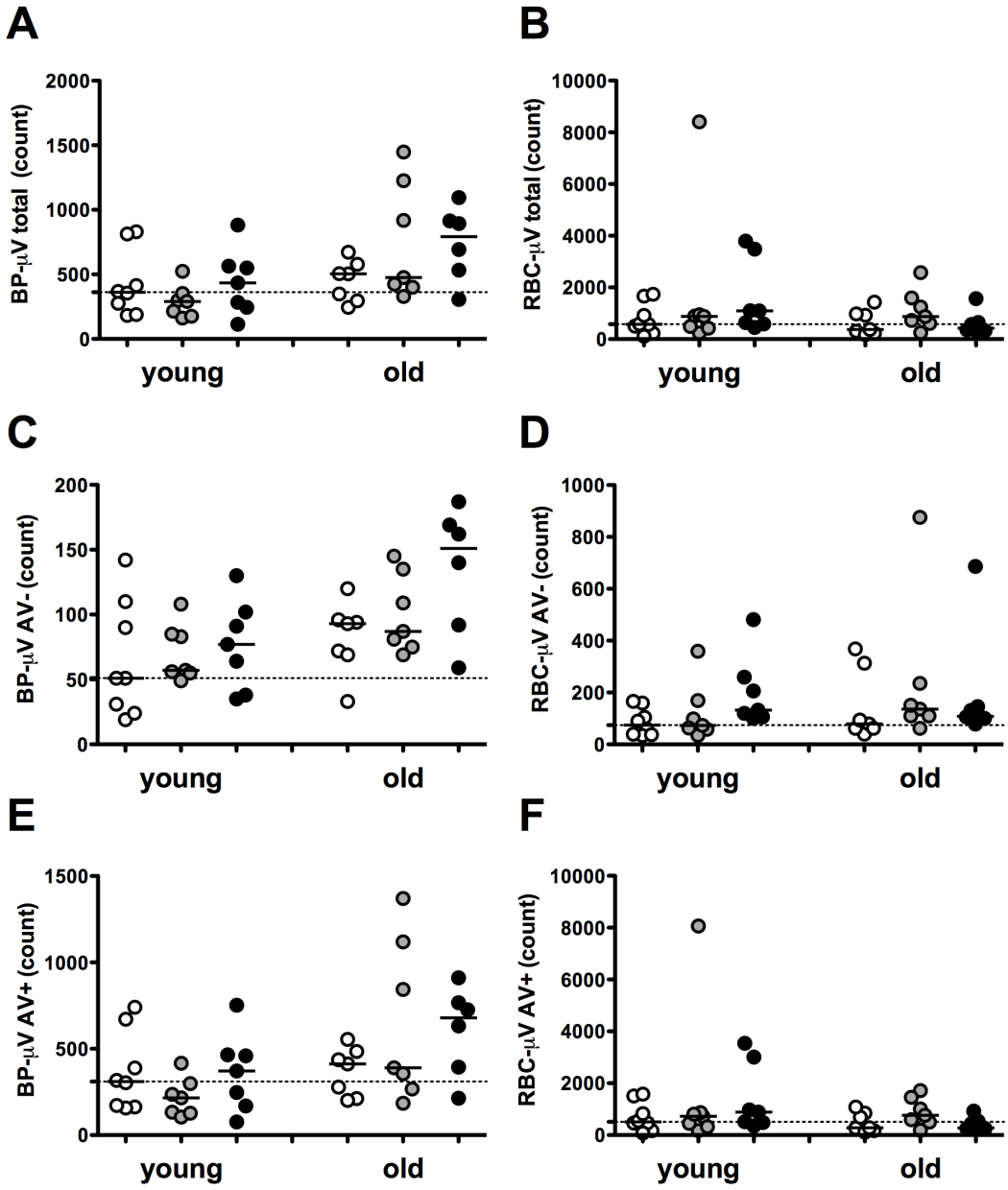

Figure 6: Microvesicle numbers
(A) blood platetelet-derived microvesicles (BP-µV), (B) red blood cell-derived microvesicles (RBC-µV), (C) BP-µV that do no bind annexin V (BP-µV AV-), (D) RBC-µV that do no bind annexin V (RBC-µV AV-), (E) BP-µV that bind annexin V (BP-µV AV+) and (F) RBC-µV that bind annexin V (RBC-µV AV+), measured by flow cytometry, in plasma from young (left) and old (right) mice, placed in a clean environment ('clean', white dots), in filtered cages inside the tunnel ('tunnel filtered', grey dots) or in unfiltered cages inside the tunnel ('tunnel exposed', black dots). Data are represented as a scatter dot plot with median values. Dotted line represents baseline median value (=value for the 'clean' group) for the young mice.

Coagulation

Baseline values were higher in old vs young mice for FVIII, but not for FVII or fibrinogen, or for any of the thrombin generation (TG) parameters. ETP in the 'tunnel

filtered' group was significantly higher than in the 'clean' group for young mice, when TG was measured upon addition of both phospholipids and TF. No air pollution-induced changes were observed in any of the other coagulation parameters (table 1).

Discussion

Young and old mice were placed in an urban roadside tunnel where they were exposed to traffic-related air pollution for more than 3 weeks. Mice were put in either unfiltered or filtered cages, and results were compared to those from mice placed in a clean environment outside the tunnel. Different hemostatic responses were observed in young and old mice.

A striking and unexpected observations in this study was that subchronic air pollution exposure raised both RBC and BP numbers, over an interval as short as 25 days, both in young and old mice. In the absence of identical controls with low exposure to PM, and due to the lack of similar data in the literature, it is difficult to correctly interpret whether this finding is related to exposure to PM, raising oxidative stress in the lungs and bone marrow, resulting in enhanced hematopoiesis (24), to exposure to gaseous pollutants such as carbon monoxide or sulfur dioxide (25, 26) or to stress (e.g. caused by noise) inducing upregulation of growth factors (27, 28). Yet, a correlation between chronic air pollution exposure and BP numbers has recently also been shown in humans (22).

Air pollution-induced increases in the concentrations of sP-sel, sE-sel and circulating VWF were only observed in young mice. While sE-sel is a specific marker for endothelial activation, sP-sel and VWF can be released by both activated endothelial cells and blood platelets. The strong correlation between these three markers in the present study suggests endothelial cell activation. This interpretation is corroborated by the demonstration of higher VWF expression in pulmonary endothelium for the tunnel mice, by semi-quantitative immunohistochemical analysis of lung sections. An altered endothelial function has been demonstrated in humans exposed to diluted diesel exhaust (29, 30). Moreover, platelet-endothelial interactions in vivo can account for a series of events in which platelets are activated, upon exposure of E-sel, P-sel and VWF by activated endothelium (31). We showed before that P-sel is

implicated in arterial thrombogenicity upon intratracheal instillation of carbon nanotubes in mice (32). Subsequent secretion by activated plateletes will further raise sPsel and VWF in the plasma. The already elevated levels of sP-sel in old mice were not upregulated upon air pollution exposure. However, a panel study of 60 elderly subjects with coronary artery disease demonstrated an association between sP-sel levels and the mean ambient 1 to 5-day concentrations of $PM_{2.5}$, but not PM_{10} (33).

A potential role for microvesicles in the induction of air pollution-induced prothrombotic changes has been suggested (34-36). Although not significant, we observed here a trend towards higher levels of total BP-μV for the old mice located inside the tunnel, with the highest median values for the 'exposed' group. As opposed to the findings for the BP activation markers, no changes were observed in microvesicle numbers in the young mice. Microvesicles can promote coagulation through the surface expression of TF and negatively charged phospholipids (20), and elevated numbers of circulating microvesicles have been demonstrated in patients with VTE (37). They accumulate on stimulated vascular surfaces that express P-selectin to form a continuous supply of procoagulant factors on a growing thrombus (38). As the majority of BP-μV in the present study bound annexin V, a measure for the surface expression of negatively charged phospholipids, such as phosphatidyl serine, the air pollution-induced increase in their number reflects a procoagulant risk factor in the old mice, which was not observed in the young.

All thrombin generation measurements were performed in the presence of an excess of exogenously added phospholipids. Therefore, these assays are insensitive to the phospholipid-dependent procoagulant state observed in the old mice exposed to air pollution. Moreover, in the absence of changes in any of the measured coagulation factors, no enhancement of the thrombin generation was observed under any condition, except for a small increase in ETP for the 'tunnel filtered' group of the young mice. The increased ETP in this group could result from decreases in anticoagulant factors, such as antithrombin or protein C, markers that were not assessed in the present study.

The paucity of changes in secondary hemostasis parameters is in agreement with previous findings of our group (19), and might be explained by the absence of an

inflammatory reaction. Mutlu et al. linked pronounced procoagulant changes upon PM exposure in mice to increased IL-6 production in alveolar macrophages (39, 40). In the present study, we did not observe increased IL-6 levels in either BALF or plasma. Kilinç et al. exposed mice via intratracheal instillation to ultra-fine particles that were collected near a roadside tunnel and observed a TF-driven increase in thrombin generation at 4 hours, and a contact activation-driven increase at 20 hours (41). Differences in administration route, exposure period and composition and dose of the particles could account for the discrepancy between these results and those of the present study.

Overall, physiological outcome parameters for the 'tunnel filtered' group were unexpectedly close to those of the 'tunnel exposed' group. Several explanations may apply: first, filtering reduced the exposure of mice considerably, but not completely. Furthermore, $PM_{2.5}$ was filtered less efficently (-32%) compared to PM_{10} (-59%). In view of the major impact of ultrafine particles on cardiovascular status (11), it is not surprising that the unfiltered and filtered groups responded similarly, but differently from the non-exposed groups.

Second, we cannot exclude that differences observed between the 'clean' group and both 'tunnel' groups might, at least partially, result from other variables such as gaseous components in air pollution, noise, environmental conditions (lighting) and stress, each of which can have independent and potentially synergistic or antagonistic effects with each other and with PM. These variables were identical for both groups inside the tunnel.

In summary, this study shows that subchronic inhalation of traffic-related polluted air does not trigger overt lung inflammation, but triggers platelet and endothelial cell activation, indicating a higher risk for arterial thrombosis. It also demonstrates that hemostatic changes upon exposure to traffic-related air pollution differ between young and old mice. Old mice, with higher baseline values of the corresponding activation markers, show a weaker proportional rise upon exposure. However, the high absolute concentrations of these markers reached in exposed old mice, in conjunction with upregulated blood platelet and blood platelet-derived microvesicle numbers are indicative of a higher overall thrombogenicity in old mice, subchronically exposed to traffic-polluted air.

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GENERAL DISCUSSION

Adapted from

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and

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Dealing with the conundrum of PM-associated DVT in the absence of changes in coagulatory changes

Recent epidemiological evidence suggests an increased risk of venous thrombosis in association with exposure to air pollution (1-3). The underlying mechanisms, however remain largely unknown, and published results with regard to markers of secondary hemostasis activation are conflicting. In this thesis, we aimed to unravel how this conundrum of PM-associated DVT in the absence of a classical procoagulant phenotype can be explained. Four possible explanations can be advanced:

1) Lack of association between air pollution exposure and DVT

Initial epidemiological reports on the existence of an association between air pollution exposure and venous thrombosis were soon followed by a number of prospective cohort studies that failed to demonstrate an association: 26,450 post-menopausal women, enrolled in the Women's Health Initiative (WHI) Hormone Therapy trials, were randomized to treatment with either hormone therapy or placebo. Regardless of the treatment category, no evidence was found for an association between short-term or long-term (up to 1 year) PM exposure and VTE (4). Another prospective study in 13,134 middle-aged persons, including men and women, also provided evidence against an association between VTE and long-term air pollution exposure, as assessed by residential distance to a major road (5). It needs to be emphasized, however, that both studies were not originally designed to examine air pollution.

The aforementioned study of Baccarelli et al. also observed a lower PM-associated risk of VTE among women compared to men (1). Furthermore, what is most surprising from the results of Baccarelli et al. is not that air pollutants are associated with VTE, but the very large magnitude of the reported risk (odds ratio 1.7 per 10 $\mu g/m^3$ elevation in PM_{10}). According to this estimate, high concentrations of air pollutants would increase the risk for VTE by an even greater magnitude than several of the major established risk factors, such as estrogens, cancer, hospitalization and heterozygous factor FV Leiden. Against this knowledge, the large effect size of the reported association appears somewhat questionable (6).

However, taking into consideration that arterial and venous thrombosis share common risk factors (7), the well-established association of air pollution exposure with arterial thrombosis (8) enhances the plausibility of an association with venous thrombosis.

Our results in mice (chapter 1) showed that although acute exposure to PM promotes arterial thrombogenicity, it does not induce venous thrombosis and only marginally raises classical risk factors of venous thrombogenicity. Subchronic exposure for 25 days to traffic pollution in a road tunnel (chapter 4) did not elevate the classical coagulation parameters either.

2) Role of the exposure duration

Another explanation for the lack of positive associations between PM exposure and classical parameters of coagulation might be found in the short duration of exposure investigated, varying from a few hours to a few days, in most experimental studies (see table 1 in the introduction). While short-term PM exposure unequivocally enhances blood platelet activation, a more chronically sustained exposure appears to be necessary to induce significant changes in the coagulation cascade.

This hypothesis is corroborated by epidemiological findings in which the risk for DVT was only associated with the mean PM concentration over a one year period, and not with any shorter time-point (1). This interpretation is compatible with our first animal study in which exposure of healthy mice to a single intratracheal instillation of DEP or UPM enhanced arterial, but not venous thrombosis (chapter 1). Indeed, although some studies suggest a short-term effect by directly translocated UFP through the activation of the coagulation cascade via contact activation (9, 10), evidence seems to favor a more prominent role for inflammatory changes upon chronic PM exposure. In this context, it is of interest that the only coagulation factor for which the associations with air pollution are consistent over different studies in humans is fibrinogen (see table 1 in the introduction), an acute phase protein upregulated during inflammatory processes.

Accordingly, no proinflammatory or consistent procoagulant associations with 'current PM' were found in our epidemiological study in patients with diabetes (chapter 2), but subacute and subchronic PM exposure was associated with hsCRP and procoagulant changes in thrombin generation assays, and chronic PM exposure with elevated numbers of procoagulant microvesicles. Increased microvesicle numbers were also found by exposing mice subchronically to traffic-related air pollution, in parallel with raised markers of arterial thrombogenicity (chapter 4).

However, whether these specific procoagulant changes, found upon long-term PM exposure, measured in *ex vivo* coagulation assays, also confer a prothrombotic risk for

DVT *in vivo* was not investigated in the present thesis; this remains to be demonstrated in studies of venous thrombogenicity in mice exposed to PM for even more prolonged periods.

3) Role of microvesicles

Coagulation factors other than fibrinogen are only marginally associated with air pollution exposure (see table 1 in the introduction). We only measured increased levels of FVII and FVIII upon intratracheal instillation of unphysiologically high concentrations (100 and 200 μg/mouse) of UPM or DEP (chapter 1), and even then, increases were mild. Hence, at 25 μg/mouse, a dose representative for the daily human exposure under very polluted conditions (11), no changes were observed in any of the measured coagulation parameters. In fact, the absence of changes in 'classical' coagulation parameters (coagulation factors, aPTT, PT, D-dimers) is a consistent finding throughout this thesis, in both humans (chapter 2) and animals (chapter 1 and 4), irrespective of the exposure period.

Even under rather extreme conditions such as exposure of mice to traffic-related air pollution inside a roadside tunnel for more than 3 weeks (chapter 4), inhaled PM doses were insufficient to induce coagulatory changes: assuming a pulmonary minute ventilation of 40 mL/min for a mouse at rest, the total volume of inhaled air over the 25-day study period would have been 1440 L. Taking into account a mean concentration for PM_{10} of 94 $\mu g/m^3$ (see chapter 4), exposed mice inhaled about 135 μg of PM_{10} over the whole study period, of which about 70 μg reached the alveoli, assuming 50% alveolar deposition.

Taken together, these findings indicate that exposure-induced changes in coagulation factors, if any, are unlikely to be solely or primarily responsible for the PM-induced increased risk of DVT.

Through the expression of procoagulant proteins and lipids on their surface, microvesicles could represent an alternative risk factor for venous thrombosis. The role of microvesicles in inflammation and coagulation is depicted in figure 1. This figure illustrates the central role of the interaction between P-selectin and PSGL-1 in the delivery of monocyte-derived microvesicles, carrying membrane-bound TF. In addition, these microvesicles, as well as microvesicles derived from blood platelets,

red blood cells and endothelial cells, expose a procoagulant phospholipid surface to which coagulation factors can bind (12). Elevated numbers of circulating microvesicles have been demonstrated in patients with VTE (13, 14). In a recently published case-control study comprising 186 VTE patients and 418 healthy controls, individuals with microvesicle concentrations above the 90th percentile of the controls' distribution had a 5-fold increased riskof having had a previous VTE, as compared to those with microvesicle concentrations below the 10th percentile (14).

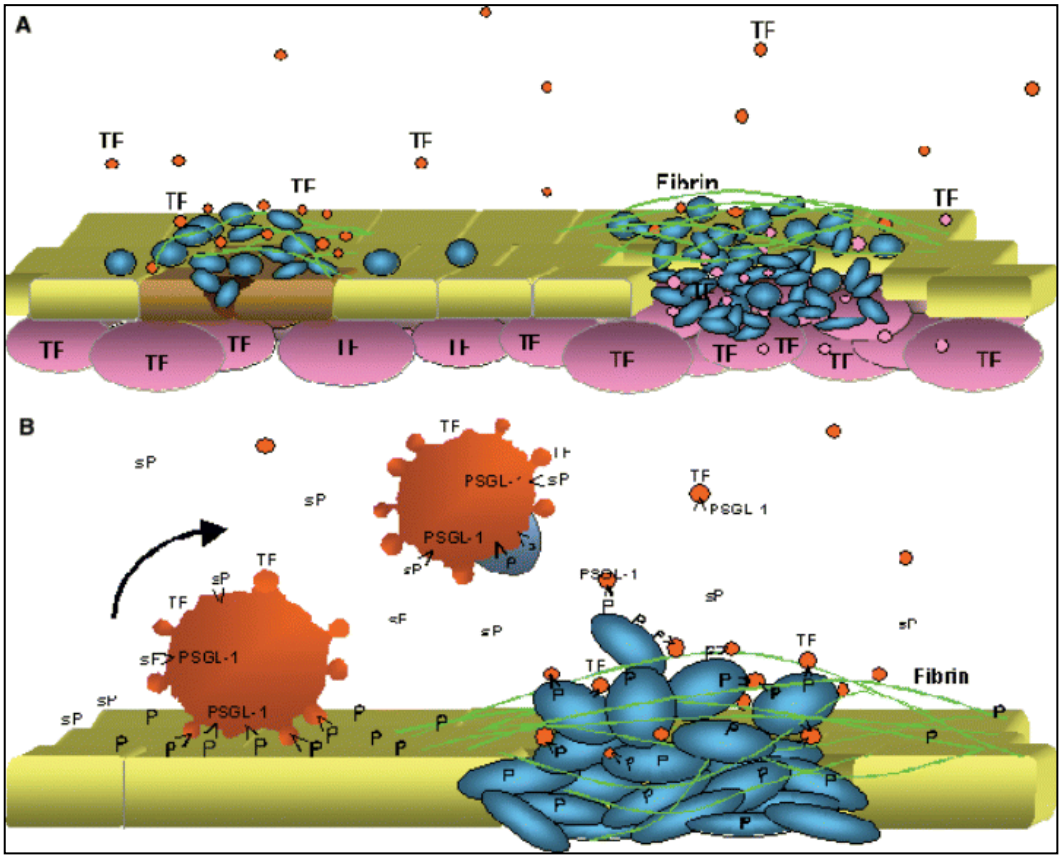

Figure 1. Role of microvesicles in coagulation.
(A) After vessel wall injury, limited to a few cells or stimulation of endothelial cells (left), subendothelial TF or TF expressed on endothelial cells initiates clotting. Platelets adhering to the site of injury with deposited fibrin promptly seal vessel wall TF from the blood stream. TF activity required for thrombus growth and stabilization is provided from blood-borne TF (orange). When large-scale damage to the vessel wall occurs (right), a significant amount of subendothelial TF enters the blood stream, lessening the importance of blood-borne TF. (B) P-selectin (P) on activated platelets and endothelial cells and P-selectin shed from these cells (sP) binds to P-selectin glycoprotein ligand 1 (PSGL-1, V) on monocytes and this induces TF-positive microvesicle generation. P-selectin on activated platelets in thrombi helps the recruitment of these microvesicles to the growing thrombus. This ultimately leads to increased thrombin generation at the site of injury (from (15) with permission).

A role for microvesicles in the induction of an exposure-associated procoagulant phenotype has been suggested by Bonzini et al., who investigated blood samples collected in steel-production plant workers. Besides shortening the PT, elevated PM

exposure also enhanced thrombin generation, but only when measured in an assay without the exogenous addition of a coagulation trigger or negatively charged phospholipids (16). These findings suggest that PM exposure may induce, as part of a systemic inflammatory response, the release of small amounts of endogenous TF (by circulating monocytes) and/or negatively charged phospholipids that may function as triggers of thrombin generation in the assay system. Circulating microvesicles might well be the source of these triggers. This hypothesis is corroborated by animal studies demonstrating elevated numbers of procoagulant microvesicles, 24 hours after intratracheal instillation of carbon nanotubes in mice (17). Likewise, when stimulated *ex vivo*, blood platelets from mice exposed to concentrated ambient PM for 2 weeks released more microvesicles relative to platelets from ambient air-exposed control animals (18).

Our epidemiological study in patients with diabetes (chapter 2) is the first to directly demonstrate upregulation of the number of microvesicles with prolonged air pollution exposure in humans. In agreement with the results of Bonzini et al. (16), enhanced thrombin generation without addition of an exogenous trigger was associated with higher subacute and subchronic levels of PM_{10}. Exposure at longer time windows, up to 1 year, elevated the numbers of microvesicles expressing procoagulant lipids.
We confirmed these findings in our last animal study, by demonstrating higher concentrations of procoagulant blood platelet-derived microvesicles in old mice subchronically exposed to traffic-related air pollution (chapter 4).

We did not investigate the exact mechanism by which contact with polluted air induces microvesicle production. However, based on our findings and those of others, we can make the following assumptions: (subclinical) pulmonary inflammation (chapter 1) and oxidative stress (19, 20) induced by inhaled PM, and possibly other constituants of air pollution, activate the pulmonary endothelium via cytokine-mediated cellular cross-talk (21). The large pulmonary endothelial bed expresses P-selectin and E-selectin on its surface, and sheds both proteins into the circulation (chapter 4). Through the expression of these and other adhesion molecules, such as ICAM-1 and VCAM-1 (chapter 1), WBC are recruited to the pulmonary vessel wall, are activated and migrate into the lung (chapter 1). P-selectin and VWF expression also support platelet rolling and adhesion to the lung, followed by platelet

sensitization (22, 23). Cross-talk between WBC and blood platelets favours further mutual activation, as evidenced by experimental studies in mice, exposed to carbon nanotubes via intratracheal instillation (17). Externalization of phosphatidylserine upon cell activation modifies the neutral membrane charge with loss of phospholipid assymetry, leading to a cascade of events which disrupt the interactions between membrane and cytoskeleton proteins, eventually resulting in the release of procoagulant microvesicles in the circulation (chapter 2 and 4). In this regard, it is striking that the association of subacute and subchronic (up to 1 month) PM exposure with the release of TF-bearing microvesicles, most likely fom monocytic origin (24), coincides with proinflammatory changes, evidenced by increased levels of CRP and fibrinogen (chapter 2).

More chronic exposure then further elevates microvesicles expressing procoagulant lipids from other sources (chapter 2), via not yet well-understood processes. Indeed, the upregulation of annexin V-binding microvesicles, associated with chronic air pollution up to 1 year, occured in the absence of increased levels of WBC, fibrinogen or CRP (chapter 2). Whether this upregulation results from direct contact with translocated particles, from oxidative stress, from direct cell-cell interactions or from other pathophysiological mechanisms remains to be investigated.

It needs to be emphasized that not all 'blood platelet-derived microvesicles' are really derived from blood platelets. In fact, throughout this thesis, we have defined 'blood platelet-derived microvesicles' as the microvesicles that are positive for the surface marker CD42a in the case of human microvesicles or CD41/61 in the case of murine microvesicles. However, in view of the large number of CD42a positive (chapter 3) or CD41/61 positive (chapter 4) microvesicles in healthy young individuals, it is unlikely that these are all derived from activated blood platelets. An alternative posibility is that microvesicles expressing platelet-specific markers are continuously derived from megakaryocytes, the precursor cells of platelets. According to this hypothesis, CD41+ or CD42+ microparticles consist of two populations, those derived from platelets following activation (by agonists, shear stress, storage or by exposure to complement proteins) or apoptosis, and those derived from megakaryocytes (25). The dynamic nature of megakaryocyte-derived microparticle formation was demonstrated by live cell microscopy (26). Inhibition of actin polymerization and stimulation of actin depolymerization augmented microvesicle

production and blebbing from megakaryocytes (26), confirming that cytoskeleton-based forces are involved in the process.

Studies that distinguish platelet-derived and megakaryocyte-derived microvesicles, based on the measurement of surface P-selectin (CD62P) expression (which is only positive on platelet-derived microvesicles), demonstrate that most (about 95%) CD41+ microvesicles in plasma are CD62P-, indicating a megakaryocytic origin (26, 27). Interestingly, these studies also demonstrated that only the CD62P+ population correlated with a history of myocardial infarction or peripheral vascular disease (27), while total CD41+ microvesicle numbers were not higher compared to healthy controls. We did not assess microvesiclular CD62P expression in the present studies. It is, therefore, difficult to conclude whether the air pollution-associated increase in platelet-derived microvesicles in mice (chapter 4) reflects enhanced blood platelet activation or results from an upregulated megakaryopoiesis. While the elevated sP-sel levels found in the tunnel exposed mice could comprise a microvesicular-derived component and therefore plead in favour of a platelet origin of CD41/CD61+ microvesicles, the strongly upregulated thrombopoiesis pleads in favour of a megakaryocytic origin (chapter 4).

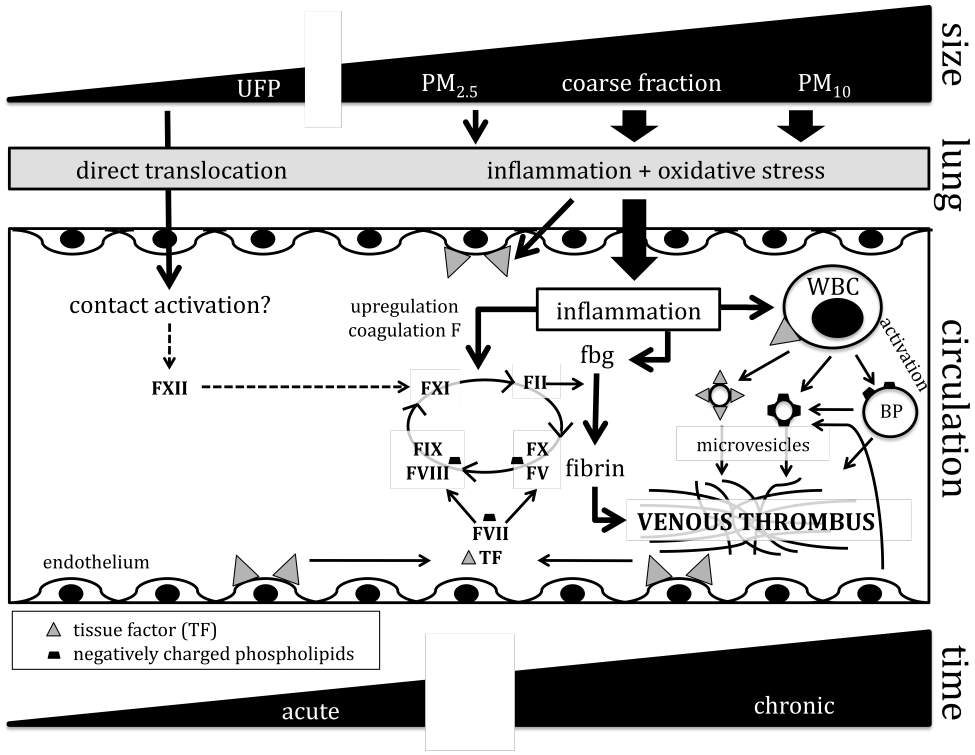

Figure 2: Biological pathways linking PM exposure and venous thrombosis
BP: blood platelet, F: factor, fbg: fibrinogen, PM: particulate matter, TF: tissue factor, UFP: ultra-fine particles, WBC: white blood cell

It is unclear how RBC are stimulated to release annexin V-binding microvesicles during these processes, but oxidative stress and shear stress, possibly induced by blood platelets bound to activated endothelial cells in the pulmonary microvasculature, have been suggested (28). A hypercoagulable state associated with enhanced RBC-derived microvesicles has been observed in a variety of diseases, such as sickle cell anemia (29), paroxysmal nocturnal haemoglobinuria (30) and transfusion complications (31). Therefore, the association between chronic (up to 1 year) air pollution exposure and RBC-derived microvesicles (chapter 2) might be of relevance to explain the link between mean annual levels of PM exposure and DVT (1). Of note, these exposure-related increased levels of RBC-derived microvesicles occurred independently of inflammatory changes (chapter 2).
Figure 2 summarizes the different pathophysiological pathways linking PM exposure to venous thrombogenicity.

4) Role of the anticoagulant pathways

If the induction of a venous prothrombotic phenotype upon air pollution exposure does not result from an upregulation of coagulation factors, it could also result from a downregulation of anticoagulatory factors such as antithrombin (AT), protein C (PC) or protein S (PS). Indeed, during inflammation, levels of anticoagulant factors decrease as the result of consumption (by ongoing thrombin generation), impaired synthesis (as a result of a negative acute phase response), degradation by elastase from activated neutrophils and endothelial dysfunction (32).
We cannot exclude that the increased thrombin generation associated with subacute and subchronic PM exposure in patients with diabetes (chapter 2) does not, at least partially, result from decreased levels of AT or activated PC (APC), because these factors were not measured in the present study. However, in a preceding pilot study conducted in 80 patients with diabetes, we did not observe any association between PM exposure and the circulating levels of AT or PC (data not shown). Likewise, intratracheal PM instillation in mice did not influence pulmonary thrombomodulin mRNA expression and even increased hepatic PC mRNA expression (chapter 1). The few other studies that investigated the anticoagulant pathways in humans as a function of air pollution exposure (see table 1 in the introduction) also failed to demonstrate any changes. However, the absence of changes in the circulating levels of anticoagulant proteins does not exclude more localized procoagulant changes at the

endothelial surface, such as altered expression of thrombomodulin or the endothelial protein C receptor (EPCR). Future studies should focus on the role of the endothelium and its anti/procoagulant status, in the lung and in the peripheral vasculature, in areas prone to development of DVT.

The elderly as a susceptible population

Elderly persons (>65 years of age) are believed to be more susceptible to deleterious cardiovascular effects of air pollution than younger persons (33). The excess in daily mortality per increment in ambient PM concentration is higher in the elderly than in the young (34-36). Hospitalization for heart failure was significantly associated with previous 14-day $PM_{2.5}$, and this effect was stronger in elderly (37). Also PM_{10} was most strongly associated with daily hospitalization for cardiovascular disease in persons >65 years old (38).

Why the elderly are more at risk than young persons is still not fully understood, but it is important to consider the concept of ‘attributable risk’.

The 'attributable risk' represents an estimate that depends both on the strength of the association between risk factor and disease and its prevalence, and is therefore defined by both baseline risk and relative risk.

The elderly have a greater baseline risk for developing VTE than young people: while the risk for deep vein thrombosis (DVT) is about 1/10,000 at the age of 40, it raises up to 1/100 at the age of 80 (39, 40) (Fig. 3).

The higher risk for VTE with ageing results from an increased prevalence of conventional risk factors, development of new, age-specific risk factors and accumulation of risk factors with age, such as immobility, malignancy, co-morbidity, hormone replacement therapy, endothelial dysfunction and a hypercoagulable state in the plasma (42). Elevation of the plasma levels of fibrinogen, homocystein, D-dimers, coagulation factors VII (FVII), FVIII and FIX and of plasminogen activator inhibitor 1 (PAI-1), have been advocated (42-45).

We confirmed these findings by demonstrating higher baseline values for FVIII, fibrinogen, VWF and TF antigen, and enhanced thrombin generation and a shortened aPTT in healthy elderly persons (chapter 3). Likewise, baseline levels of FVIII and

VWF antigen were higher in old than in young mice (chapter 4). In contrast, neither in healthy persons (chapter 3) nor in healthy mice (chapter 4) did we observe age-related differences in microvesicle numbers or their procoagulant potential.

Figure 3. Annual incidence of VTE among participants of the Worcester DVT study, by age and sex (from (41) with permission).

We assessed the relative risk related to air pollution exposure in young and old mice (chapter 4). Exposure upregulated sP-sel and VWF levels in young, but not in old mice. However, the increased values for these markers in young mice did not exceed the already higher baseline values in old mice.

While baseline values for microvesicle numbers were equal between young and old, only old mice demonstrated an exposure-related relative increase in the number of procoagulant blood platelet-derived mcirovesicles.

Taken together, these results indicate a higher attributable risk in elderly, both because of a higher baseline risk (coagulation factors, blood platelet and endothelial markers) and a higher relative risk (microvesicles). Whether these procoagulant changes also confer an enhanced prothrombotic risk for DVT remains to be elucidated in experimental studies, investigating venous thrombogenicity after subchronic and chronic PM exposure in young vs old mice.

General conclusion

A large array of epidemiological and experimental studies have provided persuasive evidence that air pollutants, the PM fraction in particular, contribute to cardiovascular morbidity and mortality. By virtue of the heterogeneity both in study design and the composition of the PM considered in these studies, it is not surprising that all findings are not totally consistent. Although evidence linking PM exposure with venous thromboembolic events is less established than for arterial events and warrants further investigation, our findings suggest that chronic air pollution exposure induces selective procoagulant changes that could elevate the risk for venous thrombosis. Inflammatory changes, along with the generation of circulating procoagulant microvesicles, seem to be more important than coagulation factor upregulation.

Both higher baseline values and pollution-associated upregulation of selected hemostasis parameters are pathophysiological mechanisms that may explain the epidemiological finding of an enhanced susceptibility to air pollution in the elderly.

Air pollution exposure may not be the highest risk factor for arterial or venous thrombosis on an individual level. However, because of the large number of persons exposed, on a global scale it is a major, and more importantly, a modifiable risk factor for cardiovascular disease and mortality.

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SUMMARY

Ambient environmental air pollutants include gaseous (carbon monoxide, nitrogen oxides, sulfur dioxide, ozone) and particulate components. Numerous epidemiological studies report consistent associations between exposure to air pollution, mainly the particulate component called 'particulate matter' (PM), and cardiovascular morbidity and mortality. Until recently, most epidemiological and experimental research has been directed towards the association between both short-term and long-term exposure to air pollution and *arterial* cardiovascular effects, such as myocardial infarction and atherosclerosis. Arterial and venous thrombosis share common risk factors. Therefore, it is not surprising that more recently, a link between chronic exposure to air pollution and *venous* thrombosis was reported.

In contrast to the well-accepted and documented deleterious effects of air pollution exposure on arterial events, data on venous events are scarce. Moreover, the mechanisms underlying the increased venous thrombogenicity remain largely unknown, and published results with regard to markers of secondary hemostasis activation are conflicting. In fact, disappointingly few studies found coagulatory changes that could constitute the basis for the observed link between air pollution and deep vein thrombosis (DVT).
In other words, the scientific community faced a conundrum of an air pollution-associated risk of venous thrombosis in the absence of well-documented changes in secondary hemostasis parameters. This formed the basis for the present thesis.

In chapter 1, we report findings of a study in mice, exposed to either urban PM (UPM) or diesel exhaust particles (DEP) via intratracheal instillation. Despite major pulmonary inflammation, which was more pronounced for UPM than for DEP, systemic inflammation was limited to increased serum IL-6 levels, 4 hours after UPM. At high concentrations, both types of PM induced similar but modest increases in FVII, FVIII and fibrinogen. Three repeated instillations did not substantially enhance the proinflammatory and procoagulant status. A single instillation of UPM enhanced experimental arterial thrombogenicity, but neither UPM nor DEP significantly enhanced venous thrombosis. Thus, a more chronic exposure seems required to increase venous thrombogenicity.

The determining role of varying time frames of exposure was assessed in chapter 2. We investigated potential associations between PM exposure, coagulation and inflammation parameters, including circulating microvesicles, in a study of 233 patients with diabetes.

Subacute and subchronic exposure to PM, estimated at the patients' residential addresses, was associated with elevated C-reactive protein (CRP), leukocytes and fibrinogen, as well as with tissue factor-dependent procoagulant changes in thrombin generation assays. When longer windows of past exposure were considered, up to 1 year preceding blood sampling, procoagulant changes were evident from the strongly increased numbers of red blood cell-derived circulating microvesicles and annexin V-binding microvesicles, but they were no longer associated with tissue factor or with inflammatory parameters. PM exposure was never associated with procoagulant changes in aPTT, PT, FVII, FVIII, FXII or D-dimers. Residential distance to a major road was only marginally correlated with increased FVIII and thrombin generation.

We conclude from these findings that increases in the number of microvesicles and in their procoagulant properties, rather than increases in coagulation factors per se, may contribute to the risk of pollution-associated VTE.

The role of microvesicles in the induction of a procoagulant phenotype, related to air pollution exposure, was further assessed in chapter 4. Subchronic (25 days) exposure to traffic-related air pollution in a roadside tunnel led to upregulated markers of endothelial and blood platelet activation (soluble P-selectin, von Willebrand factor) in young, but not in old mice. However, the increased values for these markers in young mice did not exceed the already higher baseline values in old mice. Moreover, while baseline values for microvesicle numbers were equal between young and old, only old mice demonstrated an exposure-related relative increase in the number of procoagulant blood platelet-derived microvesicles.

Higher baseline values for FVIII, fibrinogen, von Willebrand factor and tissue factor antigen, but not microvesicles, were also found in healthy elderly humans, as compared to young (chapter 3).

Taken together, these results indicate a higher attributable risk in elderly, both because of a higher baseline risk (coagulation factors, blood platelet and endothelial markers) and a higher relative risk (microvesicles), that could help to explain why

elderly are more susceptible to the deleterious cardiovascular health effects of air pollution, as observed in epidemiological studies.

Although evidence linking PM exposure with venous thromboembolic events is less well established than with arterial events and warrants further investigation, our findings suggest that (sub)chronic air pollution exposure induces selective procoagulant changes that could contribute to a higher risk for venous thrombosis. Inflammatory changes, along with the generation of circulating procoagulant microvesicles, seem to be more important than coagulation factor upregulation.
Both higher baseline values and pollution-associated upregulation of selected hemostasis parameters are pathophysiological mechanisms that may underly the epidemiological finding of an enhanced

SAMENVATTING

Luchtvervuiling bestaat uit een complex mengsel van gassen (carbon monoxide, stikstof oxiden, zwavel dioxide, ozon) en vaste deeltjes. Talrijke epidemiologische studies toonden op consistente wijze associaties aan tussen luchtvervuiling en cardiovasculaire morbiditeit en mortaliteit. Vooral de vaste deeltjes (het 'fijn stof', in het engels: 'particulate matter', PM) blijken een belangrijke impact op de menselijke gezondheid te hebben. De focus van de vroegere epidemiologische en experimentele studies lag op het verband tussen zowel korte termijn als lange termijn blootstelling aan fijn stof en *arteriële* aandoeningen, zoals acuut myocardinfarct en atherosclerose. Aangezien arteriële en veneuze trombose gemeenschappelijke risicofactoren delen, is het niet verwonderlijk dat recent een associatie tussen chronische blootstelling aan fijn stof en *veneuze* trombose werd aangetoond.

In tegenstelling tot de algemeen aanvaarde en goed gedocumenteerde nadelige effecten van blootstelling aan luchtvervuiling op arteriële aandoeningen, zijn data over het verband met veneuze aandoeningen veel zeldzamer. Bovendien is weinig geweten over de onderliggende pathofysiologische mechanismen, en spreken de studies die merkers van secundaire hemostaseactivatie onderzochten elkaar tegen. Het is in dit kader opmerkelijk hoe weinig studies procoagulante veranderingen konden aantonen die een basis zouden kunnen vormen voor de link tussen luchtvervuiling en diepe veneuze trombose (DVT).

Het uitgangspunt van deze thesis werd gevormd uit de schijnbare tegenstelling tussen een door blootstelling aan fijn stof verhoogd veneus tromboserisico enerzijds, en de afwezigheid van duidelijke veranderingen in parameters van secundaire hemostase anderzijds.

In hoofdstuk 1 worden de resultaten weergegeven van een studie waarbij muizen via intratracheale instillatie werden blootgesteld aan stedelijk stof (urban PM, UPM) of aan dieseluitlaatdeeltjes (diesel exhaust particles, DEP). Ondanks duidelijke longinflammatie, die meer uitgesproken was voor UPM dan voor DEP, bleef de systemische inflammatie beperkt tot een stijging in de serumconcentraties van IL-6, 4 uur na UPM. Aan hoge concentraties induceerden beide types van PM gelijkaardige maar milde stijgingen in FVII, FVIII en fibrinogeen. Drie repetitieve instillaties leidden niet tot een verdere opregulatie van de inflammatoire of de procoagulante

status, of slechts in beperkte mate. Hoewel de experimenteel geïnduceerde arteriële tromboseneiging werd verhoogd door een éénmalige blootstelling aan UPM, verhoogden noch UPM, noch DEP de veneuze stollingsneiging. Een meer chronische blootstelling lijkt dus vereist om een veneuze stollingsneiging te bevorderen.

De cruciale rol van verschillende tijdsvensters van blootstelling werd uitgewerkt in hoofdstuk 2. Mogelijke associaties tussen blootstelling aan fijn stof enerzijds, en stollings- en inflammatieparameters, waaronder ook circulerende microvesikels, anderzijds, werden onderzocht in een groep van 233 diabetespatiënten.
Subacute en subchronische blootstelling aan fijn stof, gemeten op het residentiële adres van alle patiënten, bleek geassociëerd met stijgingen in de concentraties van C-reactief proteïne (CRP), circulerende witte bloedcellen en fibrinogeen, en met weefselfactorafhankelijke procoagulante veranderingen in trombinegeneratie assays. Wanneer langere tijdsvensters, tot 1 jaar, in beschouwing werden genomen, konden procoagulante veranderingen waargenomen worden onder vorm van sterke stijgingen in de concentraties van circulerende rode bloedcelafgeleide en annexine V-bindende microvesikels. Op dit tijdspunt konden associaties met weefselfactor of met inflammatoire parameters echter niet langer aangetoond worden. Blootstelling aan fijn stof was op geen enkel tijdspunt geasscociëerd met procoagulante veranderingen in aPTT, PT, FVII, FVIII, FXII of D-dimeren. De afstand tussen het residentiële adres en de meest nabije grote weg correleerde slechts matig met gestegen FVIII of trombinegeneratie. Op basis van deze resultaten kan besloten worden dat stijgingen in het aantal en de procoagulante eigenschappen van microvesikels, eerder dan stijgingen in stollingsfactoren, kunnen bijdragen aan het veneuze tromboserisico, geassociëerd met subacute tot chronische blootstelling aan luchtvervuiling.

In hoofdstuk 4 werd de rol die microvesikels spelen in het induceren van een procoagulant fenotype ten gevolge van blootstelling aan luchtvervuiling verder onderzocht. Subchronische (25 dagen) blootstelling aan vervuilde lucht in een verkeerstunnel leidde tot stijgingen van merkers van endotheliale en bloedplaatsjesactivatie (plasma P-selectine, von Willebrand factor) in jonge, maar niet in oude muizen. De waarden voor deze merkers in jonge muizen stegen echter niet uit boven de reeds verhoogde basiswaarden in de oude muizen. Bovendien werden na blootstelling enkel voor de oude muizen hogere waarden gemeten voor

bloedplaatjesafgeleide procoagulante microvesikels, ondanks gelijke basiswaarden voor jong en oud.
In vergelijking met jonge gezonde vrijwilligers, werden bij gezonde ouderen ook hogere basiswaarden gemeten voor FVIII, fibrinogeen, von Willebrand factor, weefselfactor antigen, maar niet voor microvesikels (hoofdstuk 3).
Deze resultaten wijzen op een verhoging van het absoluut risico in ouderen, te wijten aan zowel een verhoogd basisrisico (stollingsfactoren, bloedplaatjes- en endotheliale merkers) als een verhoogd relatief risico (microvesikels). Dit kan helpen verklaren waarom ouderen volgens epidemiologische studies gevoeliger zijn aan de nadelige cardiovasculaire gezondheidseffecten van blootstelling aan luchtvervuiling.

Hoewel het verband tussen blootstelling aan fijn stof en veneuze trombose minder goed gedocumenteerd is dan de link met arteriële trombose, suggereren onze resultaten dat (sub)chronische blootstelling aan luchtvervuiling selectieve procoagulante veranderingen induceert die de basis kunnen vormen voor het verhoogde veneuze tromboserisico. Inflammatoire veranderingen en procoagulante microvesikels blijken een belangrijker rol te spelen dan stijgingen in stollingsfactoren. Zowel hogere basiswaarden als blootstellingsgerelateerde stijgingen in geselecteerde hemostaseparameters kunnen helpen verklaren waarom ouderen gevoeliger zijn aan luchtvervuiling.

SHORT CURRICULUM VITAE
AND
LIST OF PUBLICATIONS

SHORT CURRICULUM VITAE

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2006-2007	OLVZ, Aalst, Belgium	
2007-2011	UZ Gasthuisberg, Leuven, Belgium	
Research	**PhD**	
2007-2011	*Location:*	CMVB, K.U.Leuven, Belgium
	Subject:	Procoagulant changes in air pollution
	Promotor:	Prof. Marc F. Hoylaerts
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LIST OF PUBLICATIONS

International peer-reviewed publications:

Jacobs L, **Emmerechts J**, Mathieu C, Hoylaerts MF, Fierens F, Hoet PH, Nemery B, & Nawrot TS. Air pollution related prothrombotic changes in persons with diabetes. Environ Health Perspect 2010; 118: 191-196

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AWARDS

CSL Behring Heimburger Award, Marburg, Germany, April 2011

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